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Selective isolation of linear plasmids from *Streptomyces* spp. by concentration at a phenol-buffer interface

by

Frank Gerhard Behrend

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of
Master of Science

in
Microbiology and Biotechnology

Department of Biological Sciences

Edmonton, Alberta

Spring 1998

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Dedication

I dedicated this thesis to my parents for their unwavering support for me for everything I have done.

Thank you so very much

Abstract

A method has been developed to isolate linear plasmids of the protein-capped type from the interface of phenol extractions. This method was refined using pSCL1 of *Streptomyces clavuligerus*, a fully sequenced plasmid, as a model linear plasmid. Extraction of cell lysates under a variety of conditions demonstrated that pSCL1 could be concentrated at the interface, with selective removal of proteins and non-protein-linked nucleic acids. Several reagents were tested to determine the most effective agent for releasing the holoplasmid from the ethanol precipitated interface material and leaving it in solution. Urea proved the most successful for recovery of the protein-capped plasmid. Digestion of the precipitated interface material by proteinase K in the presence of SDS released the protein-free DNA efficiently. Most of the proteins in cell lysates were shown to partition into the phenol phase during extraction, explaining this reagent's well known success in preparing DNA and RNA without major losses due to enzyme digestion.

This method was tested on other *Streptomyces* species to show that it could be applied to a broader spectrum of organisms. It was also used in attempts to isolate the previously undetected plasmid SLP3 of *Streptomyces lividans*.

The terminal protein of pSCL1 still remains unidentified. The bond between the protein and DNA appears to be somewhat labile and the DNA-protein conjugate does not survive heating, as shown by SDS-polyacrylamide gel experiments. An approach to the isolation of linear plasmid terminal proteins is proposed, based on the use of the phenol method.

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Table of Content

	Page
1. INTRODUCTION	1
1.1 General Overview	1
1.2 *Streptomyces* linear plasmids	6
1.3 Replication	15
1.4 Phenol	27
1.5 Focus of Study	29
2. MATERIALS AND METHODS	32
2.1 Materials	32
2.1.1 Enzymes, Fine Chemicals, and Supplies	32
2.1.2 Bacterial Strains, Culture Conditions	32
2.2. DNA Isolation and Manipulation Techniques	33
2.2.1 DNA Isolation Techniques	33
2.2.2 Sucrose Gradient Separation and Purification of Plasmids	36
2.2.3 Agarose Gel Electrophoresis of DNA Samples	37
2.2.4 Restriction Endonuclease Digestion Techniques	38
2.2.5 Recovery of DNA embedded in Agarose Gels	38
2.3 Protein Analysis	38
3. RESULTS	39
3.1 Phenol-extraction method development	39
3.2 Phenol-extraction method applied to other species	49
3.3 Terminal protein analysis	59
4. DISCUSSION	72
5. BIBLIOGRAPHY	93

List of Tables

Table	Description	Page
Table 1	Summary of selected linear plasmids found in a number of different *Streptomyces* species	9

List of Figures

Figure	Description	Page
Figure 1	Physical map of pSCL1	14
Figure 2	Diagrammatic representation of bidirectional DNA replication mechanism	17
Figure 3	Diagrammatic representation of protein-primed DNA replication	20
Figure 4	Diagrammatic representation of hybrid protein-primed bidirectional DNA replication	24
Figure 5	Agarose gel (0.4%) electrophoresis of DNA extracted from *S. clavuligerus*	41
Figure 6	Agarose gel (0.4%) electrophoresis of DNA from *S. clavuligerus* cell lysate extracted with phenol/chloroform (1:1, v/v)	43
Figure 7	Agarose gel (0.4%) electrophoresis of DNA from *S. clavuligerus* extracted using pure liquefied and equilibrated phenol as well as phenol/ chloroform	46
Figure 8	Agarose gel (0.4%) electrophoresis of *S. clavuligerus* interface solubilized using different solubilizing agents	48
Figure 9	Agarose gel (0.4%) electrophoresis of DNA from *S. jumonjinensis* NRRL5741	52

Figure 10	Agarose gel (0.4%) electrophoresis of DNA from *S. jumonjinensis* ATCC 29864	54
Figure 11	Agarose gel (0.4%) electrophoresis of DNA from *S. lividans* strain 1326	56
Figure 12	Agarose gel (0.4%) electrophoresis of *S. lividans* strain 1326 interface DNA redissolved using different solubilizing agents	58
Figure 13	Agarose gel (0.4%) electrophoresis of DNA from *S. lividans* strain TK20	61
Figure 14	Agarose gel (0.4%) electrophoresis of DNA from *S. lividans* strain TK19	63
Figure 15	Polyacrylamide gel electrophoresis (12% SDS-PAGE) of *S. clavuligerus* cell lysate extracted with phenol	66
Figure 16	Polyacrylamide gel electrophoresis (12% SDS-PAGE) of *S. clavuligerus* cell lysate extracted with phenol-chloroforms (1:1, v/v)	68
Figure 17	Interface isolated pSCL1 digested with *Asp*718I	70
Figure 18	Diagrammatic representation of possible molecular content of aqueous and phenol phases after phenol extraction of cell lysate followed by centrifugation to separate the two layers	74
Figure 19	Diagrammatic representation of the 5' overhang, left by *Asp*718I, fill in reaction with $\alpha[^{32}P]$ dCTP	83

List of Abbreviations

5' dCMP	5' deoxycytidine monophosphate
[^{32}P]-dCTP	radioactive α[^{32}P] deoxycytidine triphosphate
AT	adenosine and thymidine bases
β-OH	β-hydroxyl
bp	base pair
C-terminus	carboxy terminus of a polypeptide
ccc	covalently closed circular
Da	Dalton
dATP	deoxyadenosine triphosphate
dGTP	deoxyguanosine triphosphate
DNase I	deoxyribonuclease I (bovine pancreatic)
ds	double stranded
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
GC	guanosine and cytidine bases
GLP	giant linear plasmid
kbp	one thousand base pairs
kDa	one thousand Daltons
Mbp	one million base pairs
MDa	one million Daltons
ORFs	open reading frames
oriC	bidirectional origin of replication
RNase	bovine pancreatic ribonuclease A
SDS	sodium dodecyl sulfate
SDS-PAG	sodium dodecyl sulfate polyacrylamide gel
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
ss	single stranded
TE	Tris-EDTA buffer
TEA	Tris-EDTA acetate buffer
TGS	tryptic soya glycerol sucrose medium

TIR	terminal inverted repeat
TP	terminal protein
v/v	volume per volume

1. INTRODUCTION

1.1 General Overview

Genetic information in all cells is stored in the form of DNA in the chromosomes and plasmids. All essential genes required for the survival of the organism are normally encoded on the chromosomes. The size, number, and shape of the chromosomes vary with the organism. Eukaryotic organisms usually have more than one linear chromosome. This is in contrast to most prokaryotic organisms which, as in the case of *Escherichia coli*, have a single, circular DNA molecule for a chromosome. However, there are notable exceptions in which the organism has a segmented genome consisting of two or more elements. Such is the case in *Brucella melitensis*, which has a genome divided into two circular, self-replicating molecules of different sizes, 2.1 and 1.15 Mbp (Michaux *et al.*, 1993). Also, *Rhodobacter sphaeroides* appears to have a genome that is composed of two circular and distinct chromosomes, 3 Mbp and 0.9 Mbp in size (Suwanto and Kaplan, 1989). *Agrobacterium tumefaciens* has a combination of a linear and a circular chromosome (Allardet-Servent *et al.*, 1993). Other genera, such as *Borrelia*, have a linear chromosome of approximately 1 Mbp (Ferdows and Barbour, 1989; Casjens and Huang, 1993; Fraser *et al.*, 1997) instead of the typical circular chromosome of *E. coli*. Members of the genus *Streptomyces*, as well as other actinomycetes (Crespi *et al.*, 1992), also have a linear chromosome which, in the case of certain *Streptomyces*, is 8 Mbp in size (Leblond *et al.*, 1993; Lin *et al.*, 1993; Lezhava *et al.*, 1995; Leblond *et al.*, 1996). These examples show that the generalizations which previously have been made are no longer valid

and that each genus should be examined without preconceptions about its genetic organization.

Genetic information is also encoded in plasmids. These are extrachromosomal elements that are usually non-essential for the survival of the organism. Information stored on these elements is often associated with fertility, as with F plasmids in *E. coli*, infectious antibiotic resistance, as found in the R plasmids (resistance transfer factors), or other environmental advantages such as heavy metal resistance (Silver and Phung, 1996 for a review).

To date a number of different conformations for plasmids have been discovered. The most common one is the covalently closed circular (ccc) double-stranded (ds) DNA type, such as ColEI. This plasmid is normally found in a supercoiled form *in vivo*. This topological conformation is probably universal among the ccc plasmids. The ccc dsDNA plasmids have been found throughout the bacterial world as well as in some lower eukaryotes such as the 2 μm circle plasmids in *Saccharomyces cerevisiae*.

Even though circular plasmids appear to be the norm among prokaryotic organisms, a number of linear plasmids have been discovered in a variety of bacteria. These extrachromosomal elements can be classified according to their termini.

A novel type of linear DNA molecule is that found in the plasmids and chromosome of the genus *Borrelia* (Barbour and Garon, 1987; Ferdows and Barbour, 1989, Fraser *et al.*, 1997). These have been determined to be linear dsDNA molecules with covalently closed ends. The best studied of these is the 16 kbp plasmid of *Borrelia burgdorferi* Strain B31, now completely sequenced (Barbour *et al.*, 1996), which has telomeric ends made up of an AT

rich terminal inverted repeat, or TIR, 18 bp in length, followed by four unpaired nucleotides. This conformation forms a palindrome at both ends which stabilizes the telomeres, with the unpaired bases forming the actual loop. The telomere is important for complete replication of the plasmid, and also the chromosome. Recently, in an effort to sequence the chromosome of *B. burgdorferi* strain B31, at least 17 linear and circular plamids were identified, of which 9 of the linear plasmids could be sequenced (Fraser *et al.*, 1997). Other examples of palindromic, covalently closed termini occur in the pox viruses (Baroudy *et al.*, 1982), N15 prophage in *E. coli* (Svarchavsky and Rybchin, 1984), African swine fever viruses (Gonzalez *et al.*, 1986), a fungal mitochondrial linear plasmid (Miyashita *et al.*, 1990), and a few mitochondrial DNA molecules in yeast (Dinouel *et al.*, 1993).

The other linear plasmid type is the one represented by some plasmids found in *Streptomyces*. These plasmids have a protein covalently attached to the 5' end of each strand of dsDNA and appear to be similar in structure to the adenoviruses and a number of bacteriophages, including ϕ29 *Bacillus* phage. Certain single-stranded (ss) RNA viruses, in particular the picornaviruses, also have a protein covalently linked to the 5' end (Lee *et al.*, 1977). The protein-capped linear plasmids have been found predominately in the genus *Streptomyces* of the actinomycetes group and range in length from 11.7 kbp (Keen *et al.*, 1988) to 1.7 Mbp (Kinashi *et al.*, 1993). The protein is covalently attached to the DNA via a phosphodiester bond involving the 5' phosphate and the beta-hydroxyl (β-OH) group of either a serine or a threonine or the ring hydroxyl of a tyrosine.

Another feature of these plasmids is the terminal inverted repeats (TIRs). TIRs are located at the ends of the DNA in the linear plasmids. These can vary

in size from 44 bp in SLP2, a 50 kbp plasmid in *Streptomyces lividans* 1326 (Chen *et al.*, 1993), to 80 kbp in SCP1, a 350 kbp giant linear plasmid in *Streptomyces coelicolor* (Kinashi and Shimaji-Murayama, 1991). The function of the TIRs is not completely known, but it has been suggested that they are involved in formation of secondary structures in the DNA molecule which will allow either for regulatory functions, stability of the plasmid, or both. The TIRs may also be required for replication (Sakaguchi, 1990). The protein itself is probably necessary for the complete replication of the genome, as is the case with ϕ29 *Bacillus* phage and the other viruses with this structure, where they are required to prime replication.

Linear plasmids have also been found in other species. *Rhodococcus* sp. wildtype strain MR11 has been found to harbour three linear plasmids, pHG201 (270 kbp), pHG202 (400 kbp), and pHG203 (420 kbp), (Kalkus *et al.*, 1990). As well, a second wildtype strain, MR22, has also been found to have three linear plasmids, pHG204 (180 kbp), pHG205 (280 kbp), and pHG206 (510 kbp), (Kalkus, *et al.*,1990). Two of these plasmids were found to be responsible for autotrophy characteristics of the particular *Rhodococcus* sp. wildtype strain, pHG201 and pHG205 (Kalkus *et al.*, 1990; Kalkus *et al.*, 1993). The linear plasmid pFiD188 (200 kbp) from *Rhodococcus fascians* encodes the cytokinin synthase gene responsible for fasciation, leafy gall production (Crespi *et al.*, 1992). Other species have also been found to contain linear plasmids, such as the Gram-positive *Lactobacillus acidophilus* group (Hanson and Mocquat) (Roussel *et al.*, 1993). The linear plasmid found here is 50 kbp in size. *Bacillus polymyxa* SCE2 was found to have a 16 kbp linear plasmid, pFS1, which appears to confer no discernible characteristics (Rosada and Seldin, 1993). Thus, linear plasmids seem to be not just confined

to the genus *Streptomyces*, but appear in a variety of largely Gram-positive organisms.

As indicated previously, the length of bacterial linear plasmids ranges from 11.7 kbp for pSCL1 and pSJL1 (Netolitzky *et al.*, 1997) to 1.7 Mbp (Kinashi *et al.*, 1993). Linear plasmids have been categorized into two distinct groups according to their size. Small linear plasmids are usually defined as having a size of 50 kbp or less, whereas giant linear plasmids (GLPs) have a size greater than 50 kbp. This differentiation was historically based on the detection method used. Linear plasmids detectable with conventional agarose electrophoresis are considered small, whereas GLPs could only be detected using pulse-field gel electrophoresis (PFGE). The size of some of these giant linear plasmids can be as big as, or even bigger than, the chromosomes of other organisms. For example, the *Mycoplasma genitalium* chromosome is 580,073 bp in size (Fraser *et al.*, 1995) compared to SCP1' from *S. coelicolor* which is 1.7 Mbp in size (Kinashi *et al.*, 1993).

Previously there was a problem with isolating those plasmids which have terminal proteins. During many isolation procedures phenol extraction is used to remove proteins. This, however, also removes smaller protein-linked nucleic acids, including linear plasmids of this type, from the aqueous phase. Only when an extensive protein digestion step is introduced prior to the phenol extraction can such plasmids be detected using agarose gel electrophoresis methods. Large, or giant, linear plasmids require different treatment, as vigorous shaking during the actual extraction step will usually result in shearing of linear plasmids that are significantly larger than 50 kbp in size.

1.2 *Streptomyces* linear plasmids

The genus *Streptomyces* is a group of Gram-positive eubacteria that have become of interest for a number of different reasons. Species of this genus have a complex life cycle wherein a spore, under favourable conditions, will germinate and form substrate mycelium on solid medium. At a certain stage the organism will develop aerial hyphae, which will start to curl up and begin forming spores. During the growth of aerial hyphae, secondary metabolites are formed, often including a wide variety of antibiotics. This antibiotic production has made the *Streptomyces* an industrially important group.

Streptomyces also have some genetic peculiarities. The DNA has a high G+C content (70-73%), one of the highest known. The chromosome is approximately 8 Mbp in size (see above), almost double that of *E. coli*. Combined genetic and physical maps have been constructed for a number of well studied species. These maps indicate that there are two regions of the genetic map, the 3 o'clock and 9 o'clock regions in a circular map that are genetically silent. The termini of the linear chromosome are located in the 3 o'clock region of the genetic map, explaining the genetic silence of this region. Further studies need to be done on the 9 o'clock region as its genetic silence is as of yet unexplained. The origin of replication in S. coelicolor has been located to the 8 o'clock region of the map.

There are two main types of plasmids in *Streptomyces*. First are the circular plasmids, similar to the ones found in other bacterial genera. They include pIJ101 and its derivatives. These plasmids are well reviewed elsewhere

(Hopwood *et al.*, 1986) and, therefore, will not be discussed here. They are, however, important for genetic work in *Streptomyces*.

The linear plasmids are the second type of plasmids in *Streptomyces* and of particular interest, as this study focuses on these. Linear plasmids have been found in a large number of different *Streptomyces* species. Some representative examples are summarized in Table 1. Only a few have been studied in detail. Some of the characteristics associated with these plasmids include pock formation and conjugation (Hopwood and Kieser, 1993). Pocks are described as temporary inhibition in growth, resulting in a circular area of different colony morphology (Bibb *et al.*, 1977). These pocks have been suggested to be analogous to zones of lethal zygosis produced by viruses or zones of inhibition caused by bacteriocins (Kieser *et al.*, 1982). In the case of some linear plasmids, pocks have, however, been demonstrated to be due to plasmid transfer (Bibb *et al.*, 1977) and not some other, extracelluar component. For example, pock formation was the basis for the discovery of two genetic elements in *S. lividans*, SLP2 and SLP3, of which only SLP2 has been physically identified so far.

Conjugation, also a characteristic conferred on the host by some linear plasmids, appears to be a function of direct contact between *Streptomyces* mycelial filaments (Hopwood and Kieser, 1993) and not a pilus-mediated transfer, as occurs with the *E. coli* F plasmid. Transfer of genetic information is usually decreased when a plasmid has been lost from a strain (Hopwood and Kieser, 1993).

Since a large number of linear plasmids have been identified, it is important to discuss a number of these as to their physical structure, as well as the characteristics they confer on their host. Here four of the best studied

Table 1.: Summary of selected linear plasmids found in a number of different *Streptomyces* species. The plasmids are alphabetically ordered according to their name designation. Each original host organism of the plasmids is identified, as well as the length of the plasmid in kilobases. The reference where the plasmid was first reported is indicated in the last column.

Plasmid	Organism	length in kb	Reference
pBL1	*S. bambergiensis*	640	Zotchev *et al.*, 1990
pKSL	*S. lassaliensis* NRRL 3382R	520	Kinashi and Shimaji, 1987
pSA1	*S. azureus*	9*,14.5	Ogata *et al.*, 1983
pSA1	*S. avermitilis* ATCC 31267	100	Evans *et al.*, 1994
pSA2	*S. avermitilis* ATCC 31267	250	Evans *et al.*, 1994
pSCL1	*S. clavuligerus* NRRL 3585	11.7	Keen *et al.*, 1988; Wu and Roy, 1993
pSCL2	*S. clavuligerus*NRRL 3585	120	Netolitzky *et al.*, 1995
pSCL3	*S. clavuligerus*NRRL 3585	430	Netolitzky *et al.*, 1995
pSGF2	*S. griseofuscus* C581	200	Hershberger *et al.*, 1989; Solenberg and Baltz, 1991
pSGF3	*S. griseofuscus* C581	65	Solenberg and Baltz, 1991
pSGL1	*S. griseus* NRRL 3851	120	Netolitzky *et al.*, 1995
pSJL1	*S. jumonjinensis*	11.7	Netolitzky *et al.*, 1997
pSJL2	*S. jumonjinensis* NRRL 5741	17.5	Netolitzky *et al.*, 1997
pSJL3	*S. jumonjinensis*	220	Netolitzky *et al.*, 1995
pSJL4	*S. jumonjinensis*	280	Netolitzky *et al.*, 1995
pSLA2	*S. rochei* 7434-AN4	17	Hayakawa *et al.*, 1979
pSPA1	*S. parvulus* M226	650	Chen *et al.*, 1993
pSRM	*S. rimosus*	43	Chardon-Loriaux *et al.*, 1986
SCP1	*S. coellicolor* A3(2)	350	Kinashi and Shimaji-Murayama, 1991
SLP2	*S. lividans* 1326	50	Chen *et al.*, 1993

*Linearity of this plasmid is in question

linear plasmids have been selected to give a overview of what is known about linear plasmids in *Streptomyces* with respect to conjugation, pock formation, and some genes encoded on these plasmids, where applicable.

pSLA2:

The 17 kbp linear plasmid of *Streptomyces rochei* first discovered in 1979 (Hayakawa *et al.*, 1979) and was used by Chang and Cohen (1994) to determine that some linear plasmids replicate using a hybrid replication mechanism. The majority of the plasmid is replicated using bidirectional replication from an internally located origin of replication. Only approximately 280 bp of the lagging strand 5' ends are replicated using non-displacing protein-primed replication where the 3' overhangs are used as template (Chang and Cohen, 1994). In studying the origin of replication, Chang *et al.* (1996) found that replication is initiated at direct repeats, similar to the temperate bacteriophages of *Enterobacteriacae* and *Bacillus*. Two essential proteins were also discovered, a DNA-binding protein (Rep1) and a protein resembling a DNA helicase with ATPase activity stimulated by ssDNA (Rep2). Like all the other linear plasmids found and studied so far in *Streptomyces*, this plasmid contains TIRs, 614 bp in size, as well as the typical 5' covalently bound terminal proteins.

SCP1:

SCP1 is a 350 kbp linear plasmid of *S. coelicolor* (Kirby *et al.*, 1975; Kinashi *et al.*, 1987). The terminal inverted repeats of this plasmid are 80 kbp in length (Kinashi *et al.*, 1991). Genes for the methylenomycin biosynthetic pathway, along with its resistance gene, have been located on this plasmid.

This is the first, and so far the only, linear plasmid that has been proven to have antibiotic genes located on it (Kirby, 1977, 1975). Also, this plasmid codes for three spore associated proteins, Sap C, Sap D, and Sap E (Guijarro *et al.*, 1988), the functions of which are unknown. SCP1 is known to be a fertility factor in *S. coelicolor* and *S. lividans*. This plasmid is also known to integrate into the chromosome creating NF strains. These can then no longer receive new copies of the linear plasmid, but they donate chromosomal markers bidirectionally at high frequency (Hanafusa and Kinashi, 1992). A model for integration of the plasmid into the chromosome was first proposed by Sakaguchi (1990).

SLP2:

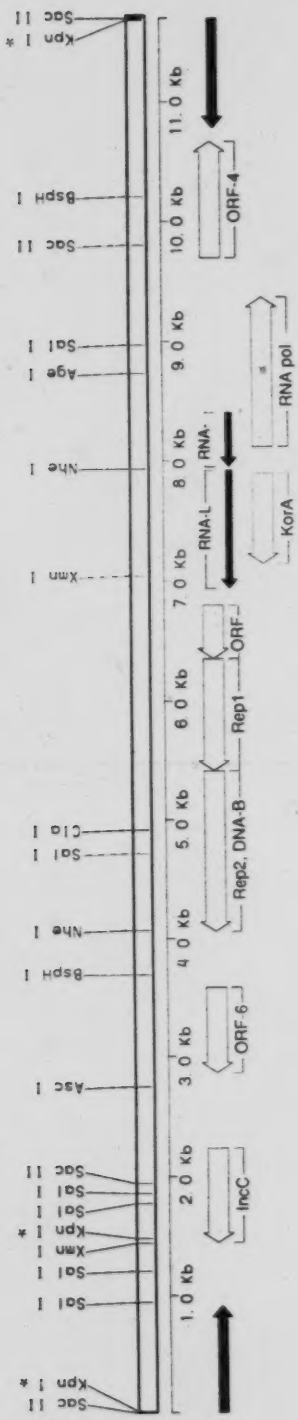
This linear plasmid from *Streptomyces lividans* has only recently been physically identified. Previously it was known to exist because of phenotypic characteristics it conferred on its host. This includes the promotion of conjugation between *Streptomyces* strains and pock formation (Hopwood *et al.*, 1983). It was found to be a 50 kbp linear plasmid which has a truncated form of Tn4811, a 5.4 kbp transposon located on it (Chen *et al.*, 1993). A second, complete copy of the transposon is present on the chromosome. This observation led to the finding that there is extensive terminal sequence homology between SLP2 and the chromosome. It provided more evidence that the chromosome is linear (Lin *et al.*, 1993), a discovery previously made by Leblond *et al.* (1993) when a physical map of the *S. lividans* chromosome was constructed. The TIRs of the plasmid were discovered to be quite short (44 bp) compared to some other plasmids.

pSCL1:

The 11.7 kbp linear plasmid pSCL1 of *S. clavuligerus* has been completely sequenced, and all of the probable open reading frames (ORFs) have been identified (Wu and Roy, 1993). A physical map is given in Figure 1 In the case of those viruses that have a similar genomic structural organization to the linear plasmids, the terminal protein, a ssDNA binding protein, and the DNA polymerase are all encoded on the genome. pSCL1 does not appear to encode any of these proteins. Of the eight possible polypeptides encoded, three (ORF-4,-5,-6) showed no significant similarities to other, known proteins. Two proteins appear to be similar to proteins encoded by pSLA2, Rep1 and Rep2, (Chang *et al.*, 1996). ORF-2 (apparent counterpart of Rep2 in pSLA2) may encode DnaB, a DNA-dependent ATPase responsible for the mobility of the primase. The apparent counterpart of Rep1, Orf-7, has characteristics of a DNA binding protein. It is interesting to note that *rep*1 is centrally located on pSLA2, as is ORF-7 of pSCL1, and contains the origin of replication, as appears to be the case with ORF-7 from pSCL1 (Chang *et al.*, 1996). The origin of replication also appears to be similar to that of some bacteriophages, such as λ (Chang *et al.*, 1996).

The protein product of ORF-L appears to be part of a regulatory system. It has sequence similarities to KorA, a component of the *kil/kor* system of pIJ101. The *kil/kor* system has been described in detail elsewhere (Hopwood and Kieser, 1993) and will not be discussed in this thesis. The ORF-1 protein product has similarities to numerous other proteins (Wu and Roy, 1993). Some of these include well known proteins such as IncC, RepA/B and SpoJ. A majority of these appear to have functions involving copy number control and/or plasmid maintenance. It is possible that ORF-1, in conjunction with

Figure 1: Physical map of pSCL1 as adopted from Wu and Roy (1993). The three *Asp*718I sites (*Kpn*I), used for the fill in experiment to help in the identification of the terminal proteins, are indicated by the asterisk. Several of the ORFs have now been assigned possible functions. ORF 1 has been labelled IncC, ORF 2 has been labelled Rep1, ORF 3 has been labelled RNA pol σ, ORF 7 has been labelled Rep2, and ORF-L has been labelled KorA. The heavy dark arrows are TIRs and the lighter dark arrows indicate detectable RNA transcripts.


Sac II
Kpn I *
11.0 Kb
BsrH I
10.0 Kb
ORF-4
Sac II
Sal I
9.0 Kb
Age I
Nhe I
8.0 Kb
RNA-
RNA pol
RNA-L
KorA
Xmn I
7.0 Kb
ORF
6.0 Kb
Rep1
Cla I
Sal I
5.0 Kb
Rep2; DNA-B
Nhe I
4.0 Kb
BsrH I
3.0 Kb
ORF-6
Asc I
Sac II
Sal I
Sal I
Kpn I *
Xmn I
2.0 Kb
IncC
Sal I
Sal I
1.0 Kb
Kpn I *
Sac II

ORF-L, is part of the maintenance mechanism of the plasmid, similar to the *kil/kor* system in pIJ101. ORF-3 resembles an RNA polymerase sigma factor. In protein database searches, about 50% of all the matches relate ORF-3 to this function. The protein product of ORF-3 may direct the cellular RNA polymerase to the plasmid to cause its transcription. With the transcription and subsequent translation of the plasmid genes by cellular enzymes, the plasmid can control replication (*rep*1 and *rep*2), maintenance (ORF-1 and ORF-L) and some other unknown plasmid activities (from one or more of the remaining ORFs, ORF-4,-5,-6).

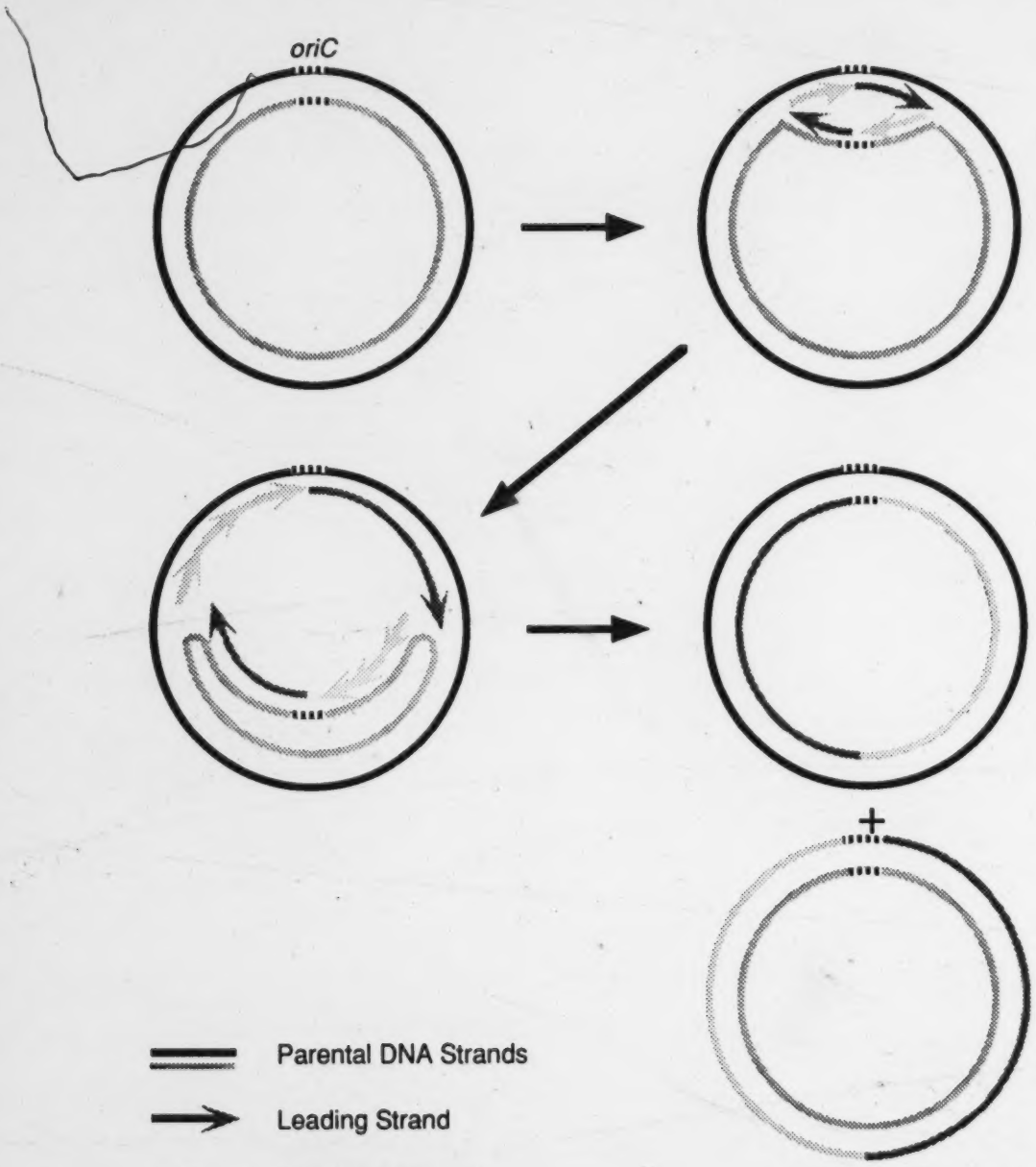
Even though this type of plasmid has been most frequently described in *Streptomyces* spp., there now appears to be a much broader spectrum of genera where this type of genetic element is found. Protein-capped linear plasmids have also been found in a *Lactobacillus gasseri* strain (Roussel *et al.*, 1993), as well as in *Paenibacillus polymyxa* (Rosoda and Seldin, 1993).

1.3 Replication

Replication of the *E. coli* chromosome has been studied in great depth and is used as a model for most other prokaryotes. Because it has been summarized sufficiently elsewhere (Kornberg and Baker, 1992, Zyskind, 1990), it will not be discussed in detail here as this is beyond the scope of this thesis. However, a brief description, as well as a diagrammatic representation (Figure 2), will be given here.

Replication starts from a fixed site, the origin of replication or the *oriC*, and preceeds bidirectionally in a 5' to 3' direction. DnaA proteins bind to each other and to the dnaA boxes of the *oriC*, causing melting of the three AT-

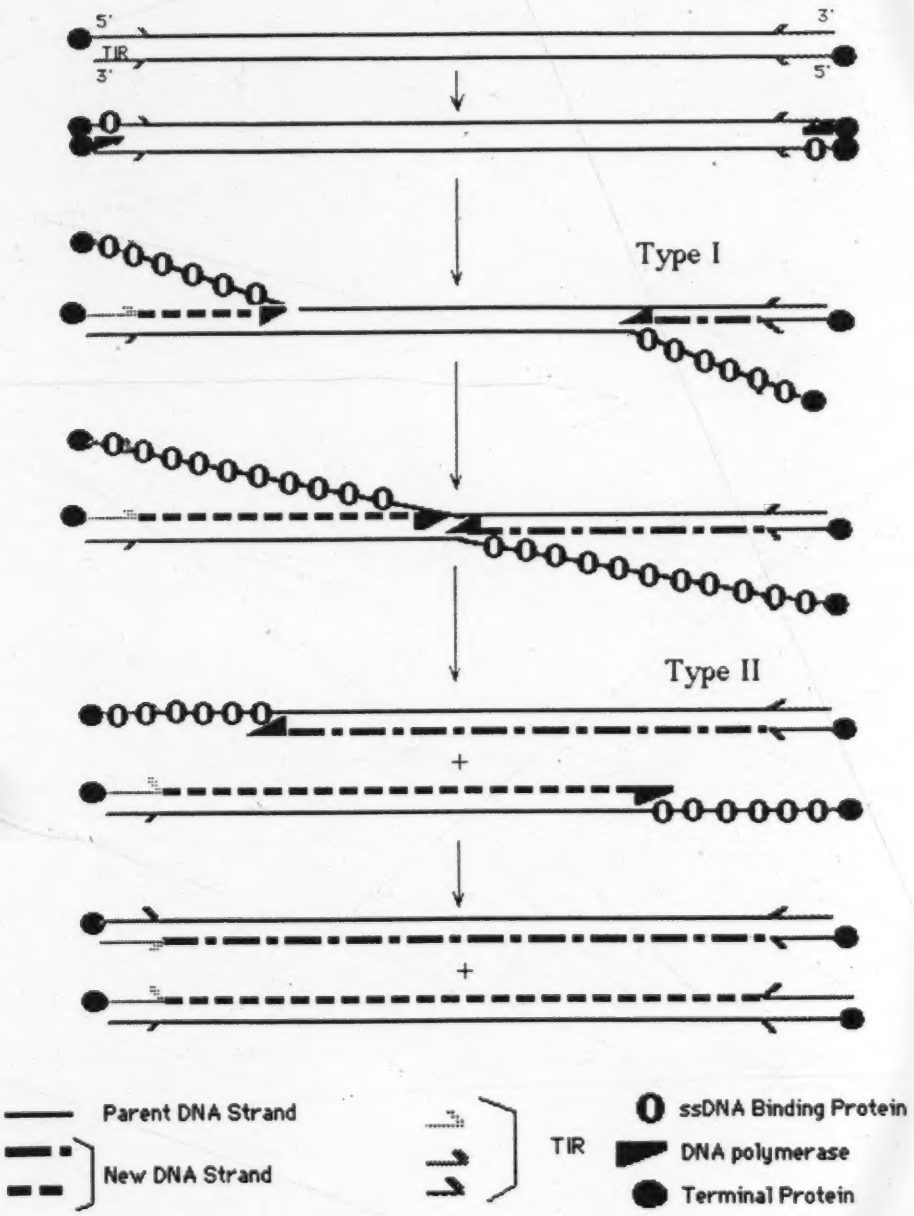
Figure 2: Diagrammatic representation of bidirectional DNA replication mechanism. The origin of circular replication (*oriC*) is indicated at the top of each circle as a different patterned band. The leading and lagging strand of DNA are represented by different patterned arrows indicating the 5' to 3' direction of replication in each. The parent strand is shown as a solid black circle and the new strand as a shaded circle.


oriC
+
Parental DNA Strands
Leading Strand
Lagging Strand
New DNA Strands in daughter chromosomes
Bidirectional Origin of Replication

rich sequences and local unwinding. This opens up the dsDNA and allows the replication enzymes to bind to the DNA and replication commences. A small fragment of RNA provides the necessary free hydroxyl (OH) group needed for the DNA polymerase to start replication. Once replication has been initiated, DNA polymerase will synthesize the new strand from the origin of replication outward in the 5' to 3' direction forming the leading strand of the replication fork. Okazaki fragments, short DNA fragments, form the lagging DNA strand where discontinuous replication must occur. The RNA is quickly replaced by DNA by the DNA polymerase and DNA ligase connects the fragments into a continuous strand. During this process, supercoiled DNA is relaxed by the activity of DNA gyrase such that DNA synthesis can continue along the molecule without any topological constraints. Once the DNA has been completely replicated, a termination sequence causes DNA polymerase to drop off the DNA strand and DNA ligase ligates the newly formed DNA strands, to form two daughter chromosomes. This replication mechanism is believed to be used for most prokaryotic chromosomes and some cccDNA plasmids.

ϕ29 is a *Bacillus subtilis* phage that has a linear dsDNA genome that is 19,285 bp long. Linear dsDNA molecules of the type of ϕ29 *Bacillus* phage have been studied extensively. Their replication process is quite well understood and has been well summarized elsewhere (Salas, 1988; Salas, 1991). A summary will be given here because of the structural similarities of the ϕ29 *Bacillus* phage to the linear plasmids in actinomycetes. The diagrammatic representation of this replication mechanism is shown in Figure 3. Each of the 5' ends of the genome is covalently linked via a phosphodiester bond to the β-OH group of a serine residue of the 28,000 Da terminal protein (TP). The TP, which is encoded on the phage genome, is important in the initiation of

Figure 3: Diagrammatic representation of protein-primed DNA replication. The terminal proteins (solid dark circle) provide the free OH needed for initiation of DNA replication and combine with the DNA polymerase I (dark, solid triangles) to form the initiation complex. DNA replication is in the typical 5' to 3' direction from the terminal ends (TIR-light patterned arrows) towards the centre forming a Type I molecule. As the replication forks meet, two Type II molecules are created. Single strand DNA binding proteins (hollow circles) prevent breakage and unfavourable interactions. Parental strands are indicated by solid lines, whereas new DNA strands are indicated by dashed lines.


5'
3'
TIR
3'
5'
Type I
Type II
+
+
Parent DNA Strand
New DNA Strand
TIR
ssDNA Binding Protein
DNA polymerase
Terminal Protein

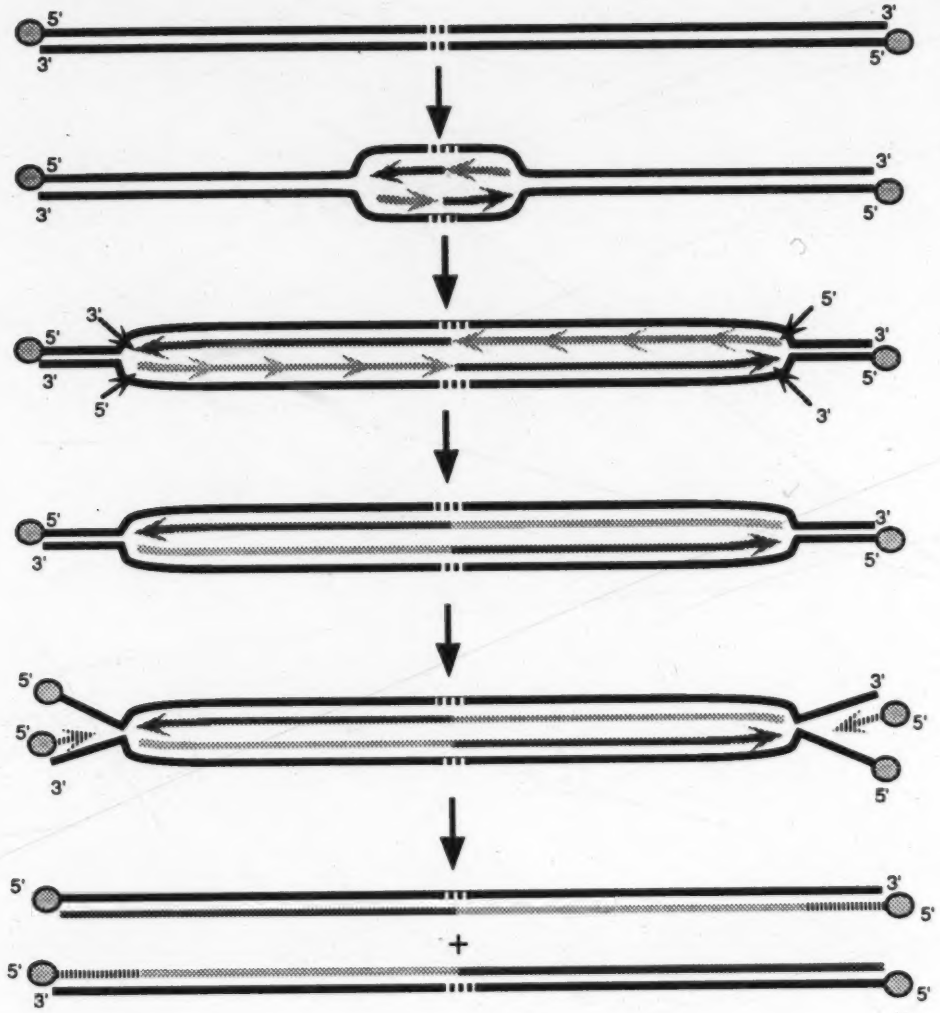
replication because it provides the free OH group needed by the DNA polymerase. There are two regions of the TP that have been identified as having potential binding domains for the polymerase. Another protein required for the replication of ϕ29 phage is protein p6, a dsDNA binding protein that usually occurs as a dimer. It can bind to two regions at each end of the genome. This binding grossly distorts the conformation of the DNA, opening the DNA double helix, allowing the TP-DNA polymerase heterodimer to bind to the DNA and the p6 protein. The second base in the genomic sequence is used as the initiation site for DNA replication. Once the first phosphodiester bond has been formed, the whole complex slips back one nucleotide, the initiation site is used once more and elongation begins.

The polymerase, encoded on the phage genome, is required for elongation. It is a highly processive enzyme and is able to move along the template strand without a requirement for any other proteins. As replication continues from both ends, a Type I DNA molecule forms. In this structure, the template segment of the DNA that has been replicated is double stranded, with the non-template strand remaining single-stranded. The unreplicated DNA remains as a double stranded molecule. When the replication forks from each end meet, the molecule separates creating two Type II molecules. This structure consists of a double-stranded replicated segment located after the replication complex and a single stranded segment in front of the replication complex to the end of the molecule. A ssDNA binding protein binds to the displaced ssDNA to protect it from nucleases and any unfavourable secondary interactions that might inhibit DNA replication. Another property of this binding protein appears to be the unwinding of DNA duplexes, such as secondary structures already formed in the ssDNA. Once the DNA has been

completely replicated, the DNA polymerase dissociates and is able to form another replication complex with a new terminal protein to go through another round of replication.

The replication of *Streptomyces* linear plasmids has not been studied in as much depth as has the replication of ϕ29 *Bacillus* phage or the adenoviruses. It was previously thought that they employed essentially the same replication mechanism. This was due to the structural similarity of the *Streptomyces* linear plasmids to the viruses in possessing a protein attached to the 5' terminus of the DNA. However, the prediction by Keen *et al.* (1988) that there may be a bidirectional origin of replication in *Streptomyces* linear plasmids, was confirmed by Shiffman and Cohen (1992). They discovered that pSCL1 could replicate when circularized, showing that it has a 'cryptic' bidirectional origin of replication and probably follows, at least in part, the basic replication mode of circular plasmids. Using this information, Chang and Cohen (1994) used pSLA2, a linear plasmid from *Streptomyces rochei*, to study replication in linear plasmids even further. They found that this linear plasmid employs bidirectional replication from an internal origin of replication. Only approximately 280 bases of the TIR are duplicated using the protein-primed mechanism seen with ϕ29 *Bacillus* phage. This ensures that the plasmid is replicated without the loss of any sequence. This hybrid replication mechanism, the protein-primed bidirectional replication mechanism, is illustrated in Figure 4. The mix of two modes of replication is unusual, if not unique. It is interesting to note, however, that this mixed mode is probably essential for *Streptomyces* when considering that it has a linear chromosome similar in structure to the plasmid. This will be addressed in more detail later in this introduction.

Figure 4: Diagrammatic representation of hybrid protein-primed bidirectional DNA replication. The centrally located bidirectional origin of replication (*oriC*) is indicated as a patterned line on the parent molecule. Bidirectional replication occurs from the center outward towards the ends as in Figure 2. The leading strand is indicated by the dark patterned line and the lagging strand by the light patterned line. Protein-primed replication occurs at the termini and moves towards the center. It is unknown whether the two modes of replications are combined sequentially as shown in this diagram.



Parental DNA

Terminal Protein

Bidirectional Origin of Replication

Leading Strand

Lagging Strand

Protein-primed Segment

The apparent absence of genes encoding a DNA polymerase and other essential proteins suggested the possibility that these might be encoded on other, not yet detected, plasmids. This led to the study by Netolitzky *et al.* (1995) and the discovery of two GLPs, pSCL2 and pSCL3, in *S. clavuligerus*, as well as GLPs in other *Streptomyces* species. It was thought that these plasmids might have the genes for the terminal protein and other replication proteins encoded on them, and that pSCL1 might be dependent on them for replication. However, it now seems probable that this is not the case. The presence of the bidirectional origin of replication, centrally located on the plasmid, suggests that the plasmid can use the cellular replication enzymes for most of its replication. Only the terminus of the lagging strand would require protein-primed replication. The essential protein that still needs to be identified is the 5' terminal protein, which protects the plasmid from exonuclease digestion and allows complete replication of the lagging strand.

The recent discovery that *S. lividans* (Leblond *et al.*, 1993), *Streptomyces griseus* (Lezhava *et al.*, 1995), and *S. coelicolor*, as well as five other species (Lin *et al.*, 1993), have a linear chromosome profoundly changes some of our ideas of *Streptomyces* chromosomes as well. It was previously thought that all, or nearly all, prokaryotic chromosomes were circular and were replicated following the *E. coli* model. Replication of the chromosome in *Streptomyces* was studied with this in mind by the search for the *oriC* on the genome. A small fragment was cloned from *S. lividans* (Zakrzewska-Czerwinska and Schrempf, 1992) that was able to replicate autonomously. This fragment contained numerous DnaA boxes with a 54% G+C content, relatively low compared to the normal content of 70-73% G+C in *Streptomyces* (Zakrzewska-Czerwinska and Schrempf, 1992). *S. coelicolor*

was also investigated to define its origin of replication. A fragment of chromosomal DNA from a cosmid appears to span the *oriC* region of *S. coelicolor* (Calcutt and Schmidt, 1992). The gene arrangement of this region closely resembles that of other eubacteria. *DnaA* and *dnaN* were found to flank a 1 kbp intergenic region containing numerous DnaA boxes. This gene arrangement seems to be highly conserved among the different bacteria, even though the intergenic region varies in size with different species. A combined physical and genetic map shows that this *oriC* is located at about the 8 o'clock region, near the centre of the linear chromosome of *Streptomyces*. Once replication is initiated from the centre of the chromosome, the replication bubble is assumed to move outward to the telomeres. As replication progresses, the essential replication genes are replicated first. This also is highly conserved among prokaryotes. Once the replication bubble moves further out, the gene arrangement is no longer conserved, showing family, genus and species variation.

Since it has been shown that the consensus region for *oriC* appears to be present in *Streptomyces*, it is likely that the linear chromosome replicates by bidirectional, *E. coli* type, replication from the origin to the telomeres. The discontinuous strand telomere is thought to be completely replicated using protein primed replication, with the 5' terminal protein providing the necessary free OH. This kind of mixed mode replication would certainly prevent certain problems from occurring during replication. For example, in pure protein-primed replication ssDNA-binding proteins are required in order to prevent breakage or endonuclease digestion of the ssDNA formed, and unfavourable binding of segments of the genome to each other. Since the linear chromosome of *Streptomyces* is approximately 8 Mbp in size, large amounts of

this protein would be required if all replication originated from the ends. It seems highly unlikely that a cell would employ a strategy requiring such a large energy expenditure during replication. A more conventional replication mode, employing a bidirectional origin as used by the circular bacterial chromosomes, is more likely to be employed. The mixed mode replication would also reduce the risk of chromosomal breakage during the replication of long stretches of single-stranded DNA. This also allows linear plasmids, both small and giant, to rely on the cellular replication proteins instead of encoding these as well as the regulatory proteins essential for their own maintenance.

1.4 Phenol

Phenol is an organic solvent that has been used in the extraction of DNA since 1956. An extraction method employing phenol was introduced by Gierer and Schramm (1956) and also by Kirby (1956). The extraction step was introduced to remove degradative proteins from cellular lysates, such as the nucleases which can seriously affect the yield of DNA and RNA. Phenol dissolves most proteins but not highly negatively charged macromolecules such as DNA and RNA. This partitioning action leads to the removal of proteins from cell lysates, leaving relatively protein-free DNA and RNA preparations, which can then be further studied. This ability of phenol to dissolve proteins is not widely appreciated. It has been frequently suggested that phenol causes precipitation of proteins at the interface between the two phases. Since the introduction of the phenol extraction procedure, minor modifications to this method have allowed very efficient DNA and RNA isolation methods to be developed.

According to Sambrook *et al.* (1989), several precautions should to be taken when using phenol extraction, including equilibration of phenol with buffer to prevent DNA partitioning into the organic phase at acid pH and also protection against oxidation of the equilibrated phenol by using anti-oxidants such as hydroxyquinoline. Hydroxyquinoline is also reported to be a partial inhibitor of RNase (Sambrook *et al.*, 1989) as well as a weak chelator of metal ions (Kirby, 1956).

Phenol is a compound that has a hydroxyl group directly attached to a benzene ring. The presence of the hydroxyl group confers some of the polar properties of alcohols to phenol, including strong hydrogen bonding. The hydrogen bonding that occurs gives phenol a much higher boiling point than toluene, which has almost the same molecular weight, 182°C compared to 110.6°C. It also is more soluble in water, 9 g/100mL H_2O at 25°C. Phenol has a $K_a = 1.3x10^{-10}$, which confers weak acid properties (Solomons, 1988), the pH of an aqueous solution is about 6.0. Conventional thinking is that this acidity can cause the DNA to be removed from the aqueous phase by partitioning into the phenol phase during phenol extractions (Sambrook *et al.*, 1989). The benzene ring portion makes phenol a fairly hydrophobic substance and therefore soluble in most other organic solvents.

Most soluble proteins in the cell are of the globular form (Lehninger, 1982). These usually have a highly hydrophobic core and a hydrophilic surface. Because of its amphiphilic properties phenol can interact with both regions of the proteins. The hydroxyl group is able to form hydrogen bonds with both the main peptide chain as well as with the polar side chains. This interaction probably disrupts some of these bonds and allows phenol to move into the inner core where it can participate in hydrophobic interactions with

the hydrophobic side chains normally sequestered in the interior of globular proteins. This largely or completely disrupts the folding of most proteins, and frequently leads to denaturation. This strong interaction with phenol forces proteins out of the aqueous phase and causes them to partition into the phenol phase. The interaction of phenol with proteins is very desirable in most cases, as this removes most of the soluble proteins and leaves the desired DNA or RNA in the aqueous solution.

1.5 Focus of Study

Previous work in this laboratory has involved the identification of pSCL1, the small linear plasmid from *S. clavuligerus* (Keen *et al.*, 1988), and its subsequent sequencing by Wu and Roy (1993). Also, a survey done by Netolitzky *et al.* (1997) identified two small linear plasmids, pSJL1 (11.7kbp) and pSJL2 (17 kbp), in *Streptomyces jumonjinensis*. In each of these studies isolation of the linear plasmids required removal of the protein using proteinase K. If the protein digestion step is not done prior to phenol extraction, the terminal protein dissolves in the phenol while the DNA remains in the aqueous phase. This localizes the DNA:protein conjugate at the phenol:water interface, effectively removing these plasmids from the aqueous phase. The plasmids concentrated at the interface are normally discarded.

No terminal protein of a linear bacterial plasmid, or linear bacterial chromosome, has yet been isolated and characterized. One approach to isolating the TP would be first to obtain the DNA:protein conjugate, which might be most conveniently done starting with the interface resulting from phenol extraction. As most, if not all, of the DNA:protein conjugates are

expected to be concentrated at the interface following such an extraction, the phenol-interface extraction method would be a powerful first step in the purification of terminal proteins. It is expected that phenol extraction would lead to the efficient partitioning of almost all cellular proteins into the phenol phase. At the same time, essentially all cellular DNA and RNA species, apart from the ones conjugated to proteins, should partition efficiently into the aqueous phase. Any DNA species trapped at the interface would most likely be linear plasmids, or telomeric fragments of linear chromosomes and giant linear plasmids, conjugated to terminal proteins. The concentrative nature of the proposed procedure should make the isolation of even low copy number linear plasmids and telomeric ends feasible.

The telomeric ends of the linear chromosome could be studied in greater depth by applying the phenol-interface extraction method. Protein-linked terminal chromosomal fragments, isolated by phenol extraction, could be digested using restriction endonucleases, followed by agarose gel electrophoresis. Only DNA molecules with defined ends would form bands in this way. This latter procedure might also benefit from an additional phenol extraction interface isolation step to remove non-terminal DNA fragments.

Removal of essentially all DNA from the terminal proteins of linear plasmids and chromosomes could be accomplished by non-specific DNases such as pancreatic DNase. Alternatively, it may be possible to use very mild alkaline treatment to effect β-elimination of the nucleic acid moiety, thus leaving the protein nucleotide-free.

When isolated in a pure state, it should be possible to determine the sequence of the TP of linear plasmids and chromosomes. From the information obtained, oligonucleotides can be designed for use as probes for detection and

isolation of the genes for the terminal proteins. Sequencing of the terminal protein genes should reveal something of their structure, as well as phylogenetic relationships with other proteins.

This thesis deals with the development of a novel method for the isolation of linear plasmids conjugated to protein. Since these conjugates were expected to be trapped at the interface during phenol extraction, material located at the interface was analyzed by electrophoresis and then subjected to various treatments to release the plasmid from the interface with the protein still attached. The *S. clavuligerus* linear plasmid pSCL1 was used as a model. Following the development of this method, a survey was done to see if it is possible to isolate linear plasmids from other *Streptomyces* species and identify previously undiscovered linear plasmids. For this survey *S. clavuligerus*, two *S. jumonjinensis* strains, and *S. lividans* strains 1326, TK19, and TK20 were examined.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Enzymes, Fine Chemicals, and Supplies

All restriction endonucleases were obtained from Boehringer Mannheim, and New England Biolabs (NEB). The Klenow fragment of DNA polymerase I, pancreatic DNase I, and DNase-free pancreatic RNase were purchased from Boehringer Mannheim. β-Agarase was obtained from NEB.

Kodak X-ray film (XAR5) was obtained from the Eastman Kodak Co. Nucleotides were purchased from Pharmacia, and [^{32}P]-labeled nucleoside triphosphates were obtained from ICN Biochemicals.

2.1.2 Bacterial Strains, Culture Conditions

Streptomyces clavuligerus NRRL 3585 (pSCL1$^+$, pSCL2$^+$, pSCL3$^+$) was obtained from NRRL, Peoria, Illinois. *Streptomyces jumonjinensis* NRRL 5741 (pSJL1$^+$, pSJL2$^+$, pSJL3$^+$, pSJL4$^+$) was also received from NRRL, Peoria, Illinois and *Streptomyces jumonjinensis* ATCC 29864 (pSJL1$^+$, pSJL2$^-$, pSJL3$^+$, pSJL4$^+$) was obtained from the American Type Culture Collection, Rockville, Maryland. Isolate information indicated that the two *S. jumonjinensis* isolates have a common origin but Netolitzky *et al.* (1995) discovered the different plasmid profile, as indicated above. *Streptomyces lividans* 1326 (SLP2$^+$, SLP3$^+$), TK19 (SLP2$^-$, SLP3$^+$), and TK20 (SLP2$^+$, SLP3$^-$; Hopwood *et al.*, 1983) were kind gifts from Dr. T. Kieser, John Innes Foundation, Norwich, UK.

All organisms were grown on tomato oatmeal agar plates (10 g tomato paste, 10 g ground oatmeal, and 12.5 g agar in 500 ml H_2O, pH 6.8) at 28°C as per Keen *et al.* (1988). Preparation of spores was conducted using the techniques described by Hopwood *et al.* (1985). For all DNA preparations mycelia were grown in TGS broth [30 g tryptic soy (DIFCO), 20 g glycerol, 15 g sucrose per liter] and incubated at 28°C on a rotary shaker at 250 rpm. Five hundred ml Erlenmeyer flasks containing 100 ml TGS broth were inoculated with spore stocks and incubated for 36-48 hours. A 1.25 cm diameter coiled stainless steel spring was placed within the 500 ml Erlenmeyer flask to aid in aerration of and the dispersal of the mycelial mass. The cultures were harvested by centrifugation at 3,800 × *g* for five minutes, followed by vacuum filtration using Whatman #3 filter paper.

2.2. DNA Isolation and Manipulation Techniques

2.2.1 DNA Isolation Techniques

The focus of this study was the development of a technique for the selective isolation of DNA molecules which have proteins covalently bound to the 5'-ends. This technique has been named the phenol-interface extraction method and is described in detail below.

Phenol-interface extraction: Liquid cultures of *Streptomyces* were harvested and resuspended in 10 ml per g (wet weight) mycelia of 25% sucrose TE Buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. Five ml of 0.25 M EDTA (pH 8.0), and 5 ml of 10 mg/ml lysozyme in 25% sucrose TE were added to each 10 ml of the above prepared suspension and incubated at 21°C for 15 to 60 minutes. *S. lividans* mycelial pellets were homogenized into smaller

fragments prior to the lysozyme treatment. Sodium dodecyl sulfate (SDS, Sigma) 20% solution was added to give a final concentration of 1% SDS. This was followed by an incubation period of up to 2 hours at 37°C. The cell lysate was then subjected to a series of phenol or phenol-chloroform extraction steps. One half volume of phenol (Fisher Scientific), either commercially available liquefied or equilibrated with buffer at a pH of 6, 7, or 8, or phenol-chloroform (1:1 mixture, v/v) was added. This was then mixed by shaking for 10 minutes, followed by centrifugation at 1,400 × *g* for 15 minutes to separate the aqueous and organic phases. Each layer, aqueous and organic, was removed separately as completely as possible, leaving up to 10% of each layer and the interface layer behind. Usually on the first extraction the upper phase constituted the organic phase whereas the lower phase was the aqueous, due to the high sucrose content. Equal volumes of TE buffer and organic solvent were added to the interface material plus the remaining aqueous and organic layers. This was followed by another cycle of 10 minutes of shaking, centrifugation at 1,400 × *g*, and removal of the organic and aqueous layers. This extraction procedure was repeated 3 to 4 times, finally reducing the volume of the aqueous and organic phases to a minimum and leaving only the interface material behind. The remaining interface material was centrifuged at 1,400 × *g* for 15 minutes in 30 ml Corex tubes and any residual aqueous and organic layer was removed. A minimum volume of TE buffer, usually 5 ml, and 1/10 of that volume of 3 M sodium acetate and two volumes of 95% ethanol were added, mixed, and DNA:protein conjugates, as well as any other remaining macromolecules, were precipitated in the freezer at -20°C for 1 hour.

Following centrifugation at 15,000 × *g* for 15 minutes, the pellet was air dried at 21°C, resuspended in a minimal volume of TE buffer, and then allowed to redissolve for up to 12 hours. To aid dissolution, a variety of solubilizing agents were added to test which of these would result in the best recovery of the DNA:protein conjugates. SDS was added to a final concentration of 0.1%. Urea was tested at a final concentration of 8 M. Guanidinium hydrochloride and guanidinium thiocyanate were tested at a final concentration of 5 to 6 M. Proteinase K in 0.1% SDS was also used to digest all proteins as a comparison for complete solubilization of the plasmid from the interface material. The resulting suspensions were placed in 1.5 ml microfuge tubes and centrifuged at 17,000 × *g* for 10 minutes, resolving the preparations into a pellet and a clear supernatant. The supernatant solutions were removed and used directly for SDS and SDS proteinase K treated samples. The supernatant of the urea treated samples was diluted 1:5 (v/v) with buffer prior to use. To the supernatants of the two guanidinium salts treated supernatants two volumes of 95% ethanol and 1/10 of a volume of 3 M sodium acetate were added to precipitate any dissolved DNA:protein complexes. These were then redissolved as completely as possible in 500 µl of TE buffer.

Two other DNA isolation techniques used in this study are described below. Their purpose was mainly a comparative one. Each of these has been given a specific name, firstly to avoid confusion among the different techniques used and secondly to provide a description of the technique in the name.

Conventional phenol-chloroform: Conventional phenol and phenol-chloroform DNA extraction is described by Sambrook *et al.* (1989) and was used to isolate cellular DNA. DNA with covalently attached proteins is selectively lost using this isolation procedure (Hirochika and Sakaguchi, 1982).

Total DNA isolation technique: Fishman and Hershberger (1983) described a method that allows the isolation of total cellular DNA using proteinase K. This is very similar to methods commonly used to isolate DNA from mammalian cells. Variations of this method have been used to isolate the DNA component of DNA:protein conjugates from other prokaryotes, as well as from viruses and plasmids of eukaryotes. In brief, the mycelia were digested with lysozyme after a wash with 25% sucrose TE buffer and resuspension in 25% sucrose TE, then treated with proteinase K and SDS. The NaCl concentration of the resulting lysate was increased to 0.5 M. The suspension was cleared by high speed centrifugation (27,000 × *g*) and this was followed by precipitation of the nucleic acids with 2-propanol.

2.2.2 Sucrose Gradient Separation and Purification of Plasmids

Purification of small linear plasmids isolated by the phenol-interface extraction method proved possible using sucrose gradient centrifugation techniques. Gradients of 13 ml (SW40 rotor) of 10%-35% sucrose in 1 M NaCl, 20 mM Tris-HCl (pH 8.0), 2 mM EDTA were produced using a two-chamber gradient maker. Plasmid containing samples of 0.1-0.5 ml were layered on top of the gradient, and then centrifuged under conditions which varied depending on the size of DNA molecules which were to be recovered. For small plasmids up to 50 kbp, samples were centrifuged for 15-24 hours at 230,000 × *g*. Gradients were fractionated by pipetting successive 0.7 ml

fractions from the top of each gradient. The DNA in each fraction was then precipitated with 0.7 ml of 2-propanol at -20°C.

2.2.3 Agarose Gel Electrophoresis of DNA Samples

DNA fragments in the range of 5 to 50 kbp were separated and their sizes determined using conventional agarose gel electrophoresis techniques (Sambrook *et al.*, 1989). Gels (0.4%) were prepared using Ultrapure Electrophoresis grade agarose (Gibco/BRL) in 1 × TEA buffer [1 × TEA contains 20 mM Tris acetate, 50 mM sodium acetate and 2 mM EDTA (pH 7.2)] in a vertical gel apparatus. Typical electrophoresis conditions ranged from 1 to 5 V/cm, for durations between 3 and 18 hours. All gels included molecular weight marker lanes, typically those produced by *Bst*EII, *Bsp*HI, *Cla*I, and *Hin*dIII digestion of λ DNA (Boehringer-Mannheim).

Low melting temperature gels (0.7%) were prepared using Low Melting Point Agarose (Boehringer-Mannheim) in 1 × TEA buffer as described above. These were used when DNA bands needed to be purified on, and later recovered from, the gels.

All electrophoresis of DNA samples isolated from *S. lividans* and *S. jumonjinensis* contained 0.1 mM thiourea which was added to the gel buffers to minimize base modification-induced DNA degradation (Zhou *et al.*, 1988, Ray *et al.*, 1992).

DNA bands on gels were visualized by illumination with ultraviolet light after staining with ethidium bromide.

2.2.4 Restriction Endonuclease Digestion Techniques

Restriction enzyme digestion of DNA in aqueous solution was conducted using the restriction enzyme manufacturer's recommended conditions and supplied buffers.

2.2.5 Recovery of DNA Embedded in Agarose Gels

pSCL1 was also purified for terminal protein analysis by cutting out pieces of low-melting point gels containing the ethidium bromide stained plasmid band. The pieces of agarose were washed twice with TE buffer (1 ml per band) for 15 minutes at 4°C, then melted at 60°C for 15-30 minutes, and incubated at 40°C with 10 units of β-agarase (New England Biolabs) for 2-3 hours. The DNA contained in this agarase digested material was used for restriction enzyme analysis after precipitation using 95% ethanol.

2.3 Protein Analysis

SDS-polyacrylamide gels with a discontinuous buffer system (Laemmli, 1970) were used to analyze the protein content of the aqueous and the organic phases and the interface material. For this, 12% polyacrylamide gels (16 × 18 cm) were prepared in a vertical apparatus and run at 20-30 mAmp for 3-4 hours as described by Sambrook *et al.* (1989). The gels were stained in 0.05% Coomassie Brilliant Blue R250 in 10% acetic acid, 25% methanol for 4-12 hours. The gels were destained in 10% acetic acid plus 25% methanol for 12-24 hours, with Kimwipes® in the destaining vessel. The gels were dried for 24-48 hours at 21°C using BioDesignGelWrap.

3. RESULTS

3.1 Phenol-extraction method development using pSCL1

The well-studied small linear plasmid pSCL1 from *S. clavuligerus* has been used for the development of a new method to isolate protein-conjugated linear plasmids. Figure 5 depicts a typical result obtained using the newly developed method. Usually a sample of the aqueous and phenol phase was removed for analysis after each extraction step. As can be clearly seen, only DNA species larger than 50 kbp are visible in one large band in the aqueous phase samples (lanes 4 to 7). No plasmid, circular or linear, is visible. The redissolved interface material shows a band at approximately 11.7 kbp, the size of the linear plasmid pSCL1, in addition to DNA fragments larger than 50 kbp in size (lane 8). The lanes containing redissolved material from the phenol phases have no, or very little, DNA present (lanes 9 to 12). As a control, total DNA prepared from an *S. clavuligerus* cell lysate using the modified Fishman and Hershberger (1983) method was run (lane 3).

As Figure 5 shows, this method appears to concentrate pSCL1 at the interface. This was accomplished using non-equilibrated, liquefied phenol. Many methods currently used, however, do not employ phenol in this way, but treat the phenol prior to use. One variation of phenol extraction uses an equal volume mixture of phenol and chloroform (Sambrook *et al.*, 1989). This mixture of organic solvents was also tested to see if similar results would be obtained. Figure 6 shows typical results obtained using this organic solvent mixture. The results appear to very similar to the ones using only phenol. Once again the material redissolved from the precipitated interface contains the linear plasmid pSCL1 (lane 8). The aqueous phases (lanes 4 to 7) show

Figure 5: Agarose gel (0.4%) electrophoresis of DNA extracted from *S. clavuligerus*. Samples of the aqueous phases of four successive phenol extractions are shown in lanes 4, 5, 6, and 7. Samples from the corresponding phenol phases were run in lanes 9, 10, 11, and 12. DNA remaining in the interface after the four extractions was run in lane 8. DNA from the modified Fishman and Hershberger (1983) total DNA preparation was run in lane 3. Molecular size markers, prepared by digestion of bacteriophage λ DNA are shown in lanes 1 (*Bsp*HI), 2 (*Cla*I), 13 (*Hin*dIII), and 14 (*Bst*EII).

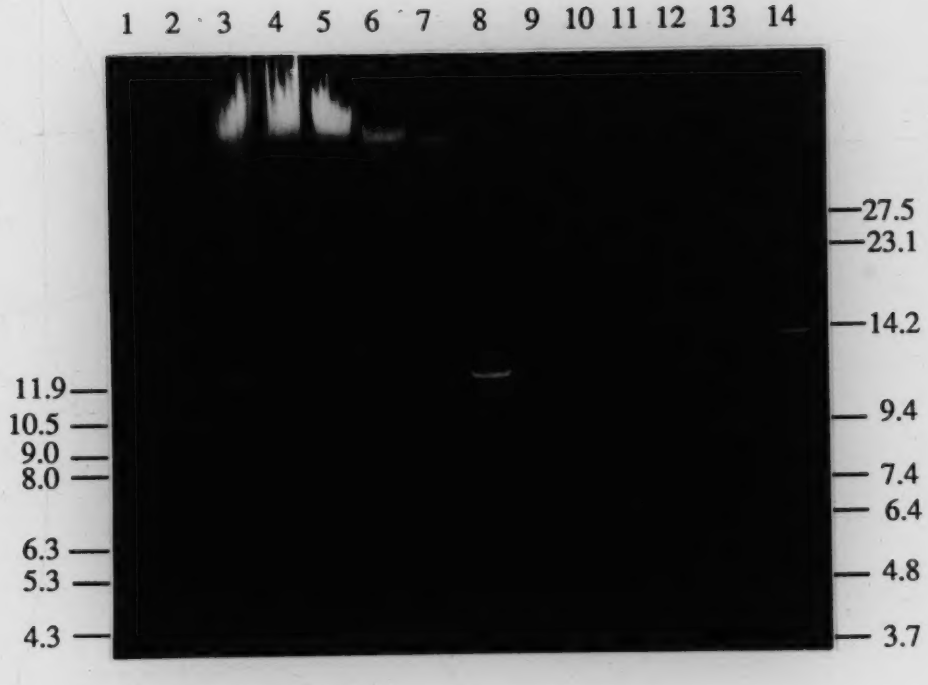
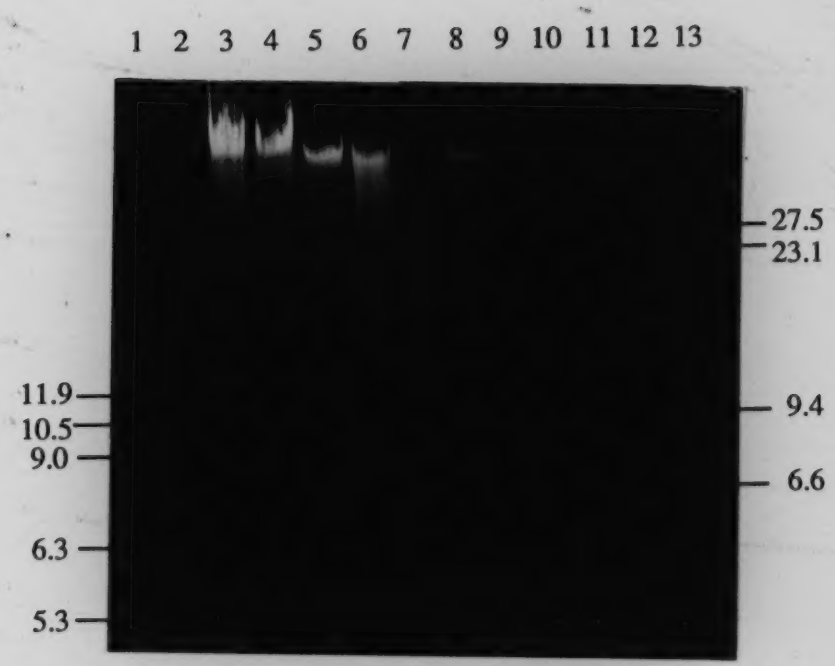

1 2 3 4 5 6 7 8 9 10 11 12 13 14
27.5
23.1
14.2
11.9
10.5
9.0
8.0
9.4
7.4
6.4
6.3
5.3
4.8
4.3
3.7

Figure 6: Agarose gel (0.4%) electrophoresis of DNA from *S. clavuligerus* cell lysate extracted with phenol/chloroform (1:1, v/v). Samples of the aqueous phases of four successive phenol/chloroform extractions are shown in lanes 4, 5, 6, and 7. Samples from the corresponding phenol/chloroform phases were run in lanes 9, 10, 11, and 12. DNA remaining at the interface after the four extractions was run in lane 8. Total DNA isolated by the modified Fishman and Hershberger method is shown in lane 3 and molecular size markers are shown in lanes 1 (*Bsp*HI), 2 (*Cla*I), and 13 (*Hin*dIII).


1 2 3 4 5 6 7 8 9 10 11 12 13
27.5
23.1
11.9
10.5
9.0
9.4
6.6
6.3
5.3

predominantly DNA species larger than 50 kbp and no plasmid. As well, the phenol phases (lanes 9 to 12) show no signs of DNA. The control lane(lane 3), containing pSCL1 from a total DNA preparation, clearly indicates that the plasmid in the interface is the 11.7 kbp pSCL1.

Once it was shown that extraction with both phenol and phenol/ chloroform could concentrate pSCL1 at the interface, it was of interest to see whether equilibration of phenol with TE Buffer, which is done frequently prior to DNA isolation methods (Sambrook *et al.*, 1989), would have any effect on pSCL1 concentration. Non-equilibrated phenol has been reported to cause an increase of DNA partitioning out of the aqueous phase (Sambrook *et al.*, 1989). Phenol was equilibrated to a pH of 6.0, 7.0, or 8.0 and a sample of cell lysate was extracted with each. Visual observation of the interface showed a decrease in the volume of the interface (not shown) as the pH of the phenol increases. Figure 7 illustrates the material found trapped at the interface in both phenol (lane 3) and phenol/chloroform (lane 4) extractions, as well as in the three equilibrated phenol interfaces(lane 5, 6, and 7 respectively). There appears to be no major difference in the amount of the DNA present at the interface.

One of the problems encountered in the development of this method was dissolving the precipitated interface material. When attempts were made to redissolve this material in TE Buffer only very little or no plasmid could be detected (not shown). This contrasts with the situation when SDS was added to a concentration of 0.1% (Figure 8). At this point it became of interest to see if other solubilizing agents could be used. These were tested to determine which would allow the best recovery of the plasmid from the interface. As can be seen in Figure 8, when SDS was added to a concentration of 0.1%, pSCL1

Figure 7: Agarose gel (0.4%) electrophoresis of DNA from *S. clavuligerus* extracted using pure liquefied phenol, equilibrated phenol and phenol/chloroform. Lane 3 shows the DNA remaining in the interface after extraction with pure liquefied phenol. Lane 4 shows the DNA at the interface after phenol/chloroform extraction. Lane 5 shows DNA remaining at the interface after extraction using phenol equilibrated to pH 6.0. Lane 6 shows the same after extraction with phenol equilibrated to pH 7.0, and lane 7 shows the DNA after using phenol equilibrated to pH 8.0. Lane 8 shows total DNA isolated by the modified Fishman and Hershberger method. Molecular sizes markers are shown in lanes 1 (λ *Bsp*HI digest), 2 (λ *Cla*I digest), 9 (λ *Hin*dIII digest), and 10 (λ *Bst*EII digest).

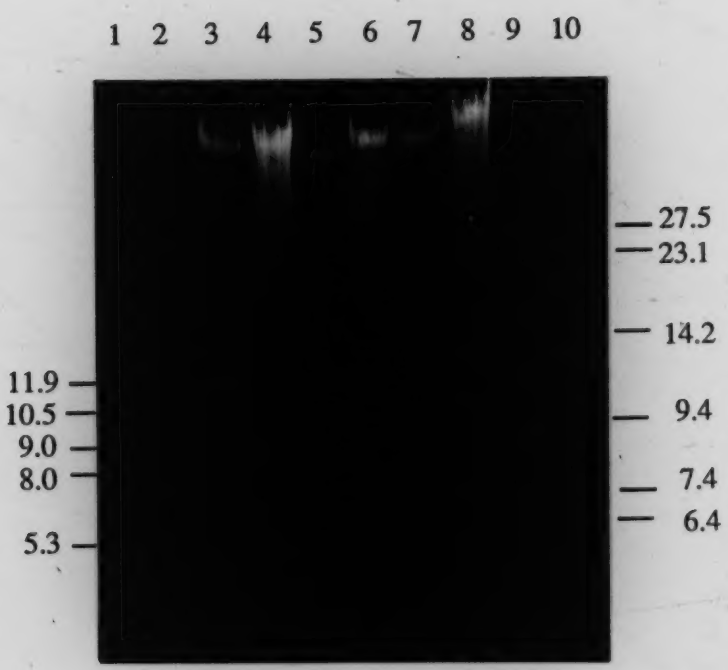
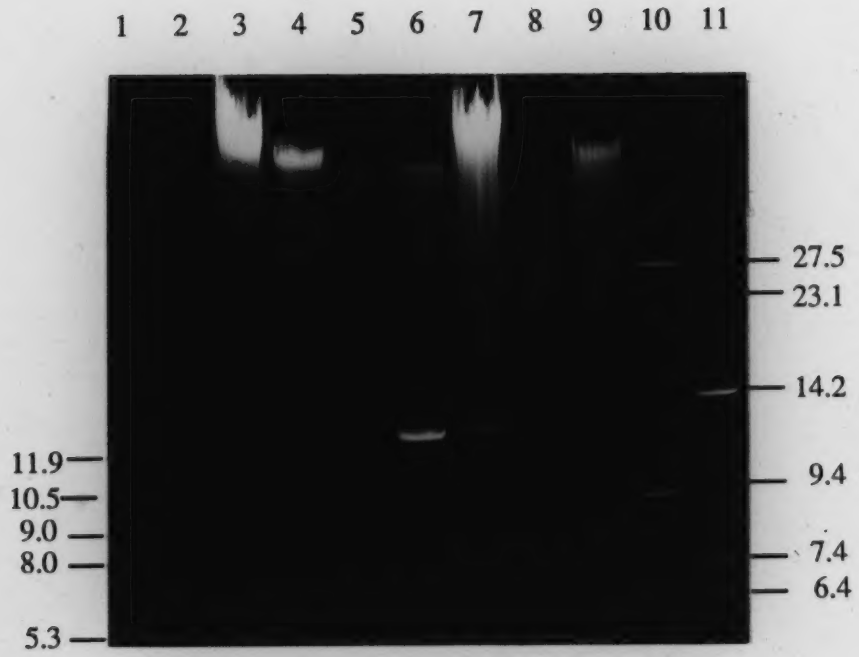

1 2 3 4 5 6 7 8 9 10
27.5
23.1
14.2
11.9
10.5
9.0
8.0
9.4
7.4
6.4
5.3

Figure 8: Agarose gel (0.4%) electrophoresis of *S. clavuligerus* interface DNA solubilized using different solubilizing agents. Lane 3 shows chromosomal DNA from *S. clavuligerus*. Total *S. clavuligerus* DNA isolated by the modified Fishman and Hershberger method is shown in lane 4. Lane 5 shows DNA from the interface solubilized in 0.1% SDS TE buffer. Lane 6 shows DNA from the interface solubilized in 0.1% SDS TE buffer and treated with proteinase K. In lane 7 interface solubilized with 8 M urea, precipitated with ethanol, redissolved in TE buffer, and diluted 1:5 in TE buffer is shown. Lane 8 shows DNA from the interface solubilized with 5 M guanidinium hydrochloride, precipitated with ethanol and redissolved in TE buffer. In lane 9 interface solubilized with 5 M guanidinium thiocyanate, precipitated with ethanol and redissolved in TE buffer is shown. Size markers are shown in lanes 1 (*Bsp*HI), 2 (*Cla*I), 10 (*Hin*dIII), and 11 (*Bst*EII).


1 2 3 4 5 6 7 8 9 10 11
27.5
23.1
14.2
9.4
7.4
6.4
11.9
10.5
9.0
8.0
5.3

could be detected. SDS-proteinase K addition increased the intensity of the pSCL1 band greatly compared to SDS only treatment (lane 6). Urea, a good hydrogen bond disrupter, was one of the redissolving agents tested. It is very effective in solubilizing large amounts of both pSCL1 and high molecular weight DNA from the interface (lane 7). The redissolved interface had to be diluted about 1:5 for the plasmid to be detectable in a background of large molecular weight DNA fragments that were released from the interface material. Guanidinium hydrochloride (lane 8) and guanidinium thiocyanate (lane 9), also good chaotropic agents, did not appear to aid the redissolving of the linear plasmid from the interface greatly. Urea, guanidinium hydrochloride, and guanidinium thiocyanate supernatants were all precipitated with ethanol prior to electrophoresis for desalting. Of all of the treatments, the latter three changed the appearance of the white interface material (not shown) most dramatically to a transparent appearance.

3.2 Phenol-extraction method applied to other species

Although this procedure seems to concentrate pSCL1 at the phenol:aqueous interface, it was important to see if this method could be applied to other linear plasmids. A number of other *Streptomyces* species are known to have linear plasmids. These vary in size from 11.7 kbp in *S. jumonjinensis* to a 50 kbp plasmid in *S. lividans* and larger. For this study, linear plasmids from *S. jumonjinensis*, *S. coelicolor*, and several *S. lividans* strains were investigated. Here once again the aqueous and phenol phases were compared with the redissolved interface and a total DNA preparation for each species as a control. *S. jumonjinensis* NRRL 5741 has two "small" linear plasmids, pSJL1 (11.7 kbp) and pSJL2 (17.5 kbp) (Netolitizky *et al.*, 1997),

which can be identified as faint bands in the redissolved interface (Figure 9, lane 7). *S. jumonjinensis* ATCC 29864 has only pSJL1 which can also be identified using the phenol-interface extraction method (Figure 10, lane 8).

S. lividans is a widely studied *Streptomyces* species that was included in this study. The wild type strain, *S. lividans* 1326, is known to have at least 2 plasmids, SLP2 and SLP3 (Hopwood *et al.*, 1983). SLP2 is a 50 kbp linear plasmid that has been identified recently (Netolitzky *et al.*, 1991, Yu *et al.*, 1991, Chen *et al.*, 1993). The characteristics of SLP3 are as yet unknown. As can be seen in Figure 11, no plasmid could be detected in the aqueous phases (lanes 8 to 10). As well, no plasmid could be found using the phenol extraction-interface method, where the interface material is redissolved using 0.1% SDS only (lane 7). When the modified Fishman and Hershberger method was used, SLP2 was clearly shown to be present (lane 3). Some unknown factor appears to prevent the detection of SLP2 using the phenol-interface extraction method even though this plasmid can be clearly seen on a conventional 0.4 % agarose gel.

To examine this further, the interface material was subjected to not only 0.1% SDS but also proteinase K digestion and 8M urea dissolution (Figure 12). A faint band running at the approximate same rate as SLP2 from the total DNA preparation can be detected in the SDS proteinase K treatment of the precipitated interface material (lane 5). This band can not be seen when either SDS alone (lane 4) or 8M urea (lane 6) is used, but it is possible that SLP2 can be isolated using the phenol-interface method.

Another *S. lividans* strain used in this survey was TK20 ($SLP2^+$, $SLP3^-$). It was expected that SLP2 might be detected in this strain, but not SLP3 since this strain had been cured of that plasmid. Figure 13 clearly shows that

Figure 9: Agarose gel (0.4%) electrophoresis of DNA from *S. jumonjinensis* NRRL5741. Samples of the aqueous phases of four successive phenol extractions are shown in lanes 3, 4, 5,and 6. Samples from the corresponding phenol phases were run in lanes 8, 9, 10, and 11. DNA remaining at the interface after the four extractions was run in lane 7. Total DNA of *S. jumonjinensis* NRRL5741 isolated by the modified Fishman and Hershberger method was run in lane 2. Molecular size markers, prepared by digestion of bacteriophage λ DNA are shown in lanes 1 (*Cla*I), 12 (*Hin*dIII), and 13 (*Bst*EII).

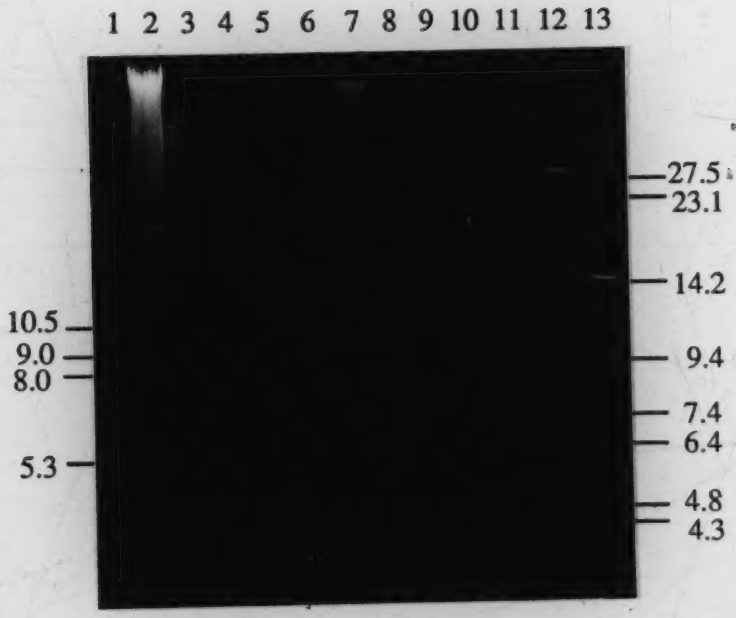

1 2 3 4 5 6 7 8 9 10 11 12 13
27.5
23.1
14.2
10.5
9.0
8.0
9.4
7.4
6.4
5.3
4.8
4.3

Figure 10: Agarose gel (0.4%) electrophoresis of DNA from *S. jumonjinensis* ATCC 29864. Samples of the aqueous phases of four successive phenol extractions are shown in lanes 4, 5, 6, and 7. Samples from the corresponding phenol phases were run in lanes 9, 10, 11, and 12. DNA remaining at the interface after the four extractions was run in lane 8. Total DNA of *S. jumonjinensis* ATCC 29864 isolated by the modified Fishman and Hershberger method was run in lane 3. Molecular size markers, prepared by digestion of bacteriophage λ DNA are shown in lanes 1 (*Bsp*HI), 2 (*Cla*I), 13 (*Hin*dIII), and 14 (*Bst*EII).

1 2 3 4 5 6 7 8 9 10 11 12 13 14
27.5
23.1
14.2
11.9
10.5
9.0
8.0
9.4
7.4
6.4
6.3
5.3
4.3
4.8

Figure 11: Agarose gel (0.4%) electrophoresis of DNA from *S. lividans* strain 1326. The aqueous phase samples and phenol phase samples were run in lanes 8, 9, and 10, and 4, 5, and 6, respectively. DNA redissolved from the interface was run in lane 7. The total DNA preparation of *S. lividans* strain 1326 is shown in lane 3. Molecular size markers were run in lanes 1 (λ *Bst*EII digest), 2 (λ *Hin*dIII digest), 11 (λ *Cla*I digest), and 12 (λ *Bsp*HI digest).

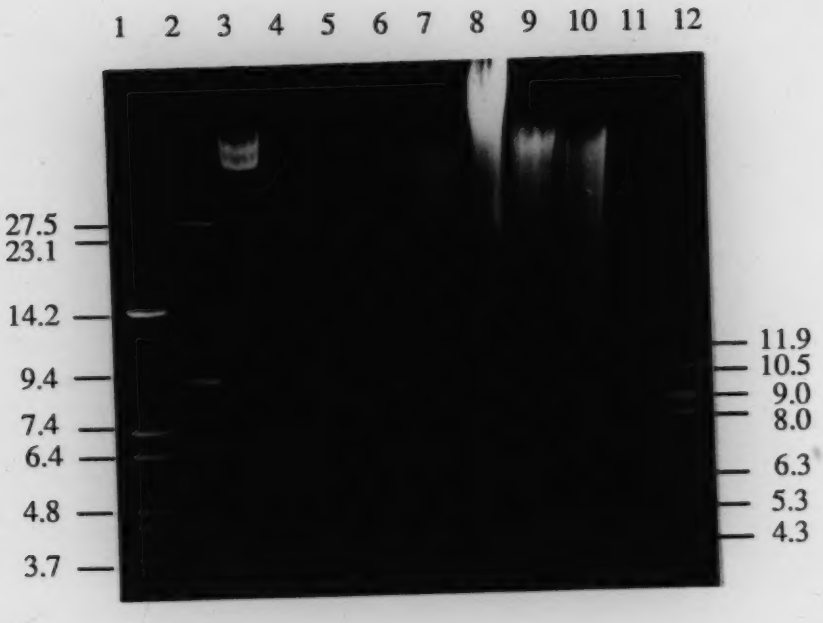
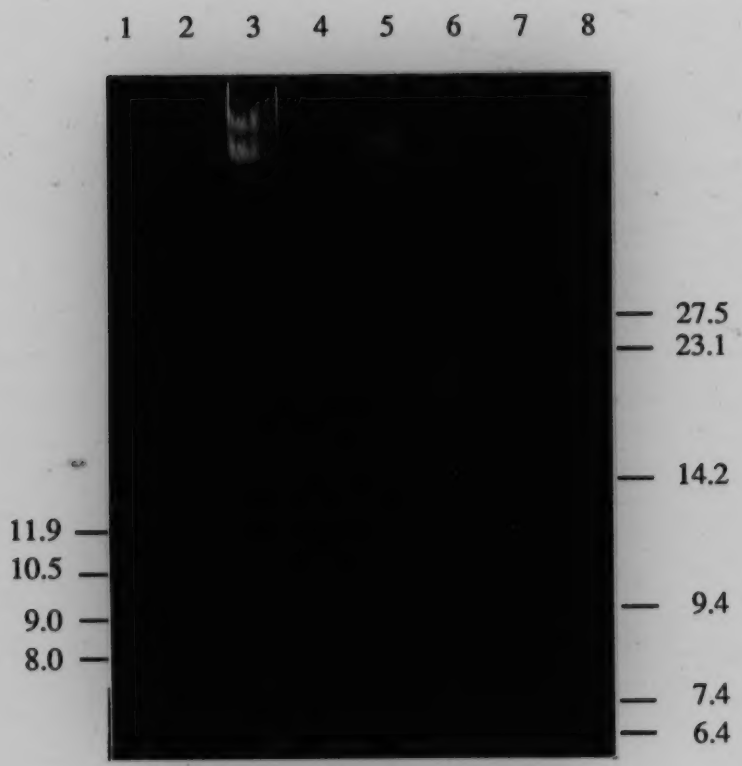

1 2 3 4 5 6 7 8 9 10 11 12
27.5
23.1
14.2
9.4
7.4
6.4
4.8
3.7
11.9
10.5
9.0
8.0
6.3
5.3
4.3

Figure 12: Agarose gel (0.4%) electrophoresis of *S. lividans* strain 1326 interface DNA redissolved using 0.1% SDS (lane 4), 0.1% SDS proteinase K (lane 5), and 8M urea (lane 6). Total DNA preparation of *S. lividans* was run in lane 3. Molecular size markers were run in lanes 1 (λ *Bsp*HI digest), 2 (λ *Cla*I digest), 7 (λ *Hin*dIII digest), and 8 (λ *Bst*EII digest).


1 2 3 4 5 6 7 8
27.5
23.1
14.2
11.9
10.5
9.0
8.0
9.4
7.4
6.4

the expected results were in fact obtained, as only SLP2 could be detected in the total DNA preparation (lane 3). Once again, SLP2 was not detected in the aqueous phases (lanes 8 to 10) or in the interface (lane 7), using only SDS for redissloving the interface material.

The last strain of *S. lividans* used was TK19 ($SLP2^-$, $SLP3^+$). This was included when the utility of the method was first demonstrated and it was believed that the previously unidentified plasmid SLP3 might finally be detected physically. Figure 14 represents the results obtained for this strain. The aqueous phases show two bands, indicating plasmids of apparent linear sizes 6.3 kbp and 11.9 kbp, respectively (lanes 3 to 5). Once again there is no DNA in the phenol phases (lanes 7 to 9). The interface (lane 6) and the total DNA preparation (lane 10) show both of the plasmids that have been isolated from the aqueous phase. In addition to these plasmids, two other bands, running slightly faster at 4.9 kbp and 9.0 kbp, can be detected. As well, there appears to be a band running in the 50 kbp range in the total DNA preparation.

3.3 Terminal protein analysis

A second aspect of this project was to investigate the terminal protein of pSCL1. Previously the isolation of linear plasmids required the use of proteinase K prior to phenol extraction. With the possibility of recovering linear plasmids from the interface of the phenol extraction the proteinase K step can be deleted. The DNA:protein conjugate could now be recovered more selectively and completely, and a way to isolate the protein might be available. To this end it was of interest to examine the protein content of the aqueous and phenol phases, and the interface. Samples from the different

Figure 13: Agarose gel (0.4%) electrophoresis of DNA from *S. lividans* strain TK20. Aqueous phase samples and phenol phase samples are shown in lanes 4, 5, and 6, and 8, 9, and 10, respectively. The DNA remaining at the interface was run in lane 7 after redissolving with 0.1% SDS. Total DNA of *S. lividans* TK20 isolated by the modified Fishman and Hershberger method was run in lane 3. Molecular size markers are shown in lanes 1 (λ *Bst*EII digest), 2 (λ *Hin*dIII digest), 11 (λ *Cla*I digest), and 12 (λ *Bsp*HI digest).

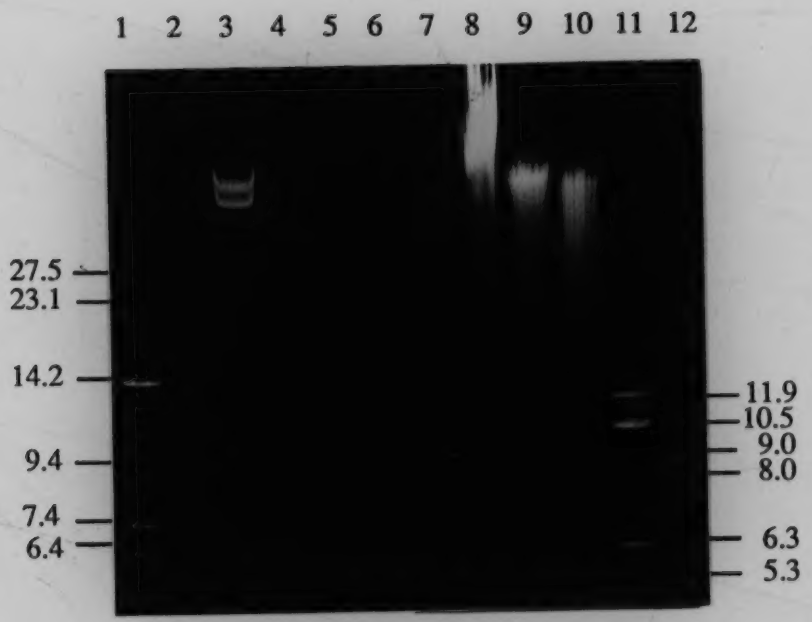
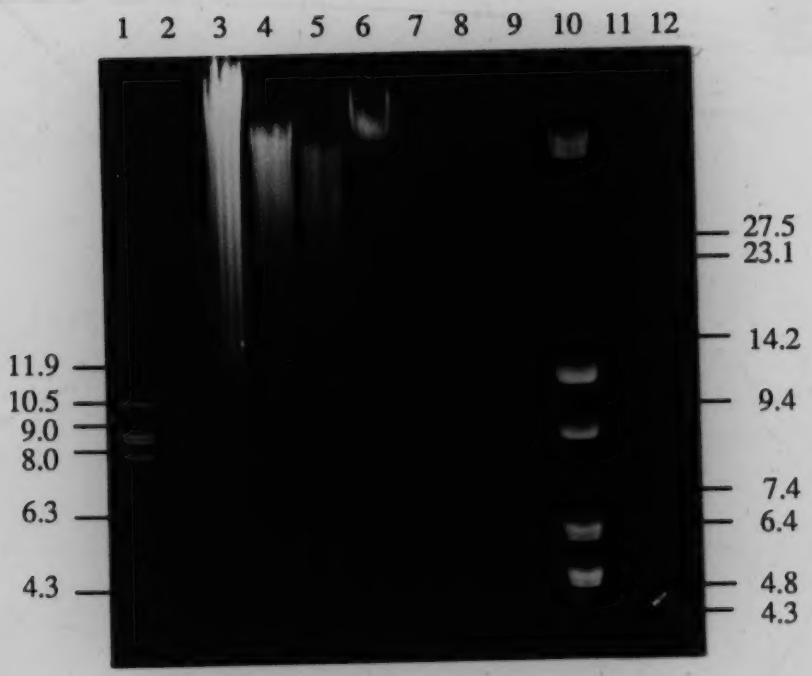

1 2 3 4 5 6 7 8 9 10 11 12
27.5
23.1
14.2
9.4
7.4
6.4
11.9
10.5
9.0
8.0
6.3
5.3

Figure 14: Agarose gel (0.4%) electrophoresis of DNA from *S. lividans* strain TK19. Aqueous phase samples and phenol phase samples are run in lanes 3, 4, and 5, and 7, 8, and 9, respectively. Interface DNA was run in lane 6. Total DNA from *S. lividans* TK19 isolated by the modified Fishman and Hershberger method was run in lane 10. Molecular size markers are shown in lanes 1 (λ *Bsp*HI digest), 2 (λ *Cla*I digest), 11 (λ *Hin*dIII digest), and 12 (λ *Bst*EII digest).


1 2 3 4 5 6 7 8 9 10 11 12
11.9
10.5
9.0
8.0
6.3
4.3
27.5
23.1
14.2
9.4
7.4
6.4
4.8
4.3

phases used in Figure 5 were run on a 12% SDS-PAGE and stained with Coomassie Blue. As can be seen in Figure 15, the four aqueous phases did not contain any detectable amount of protein. The interface appears to have little to no protein in it. The majority of the protein can be detected in the phenol phase samples, with decreasing concentrations of proteins with successive extractions. A similar gel was run using samples prepared by the phenol/chloroform treatment. The results obtained can be observed in Figure 16. The results obtained appear to be similar to the ones from the phenol only extractions. It is important to note that the majority of the proteins are, once again, in the organic solvent phases with decreasing concentrations of protein in successive extractions.

A direct approach in terminal protein isolation proved unsuccessful. The 0.1% SDS redissolved interface material, loaded on a 13 ml sucrose gradient did not yield any useful results (data not shown). Even though the plasmid was digested with DNase I prior to separation on a 12% SDS-PAGE, no protein could be detected by the Coomassie Blue stain. Instead, only bands present in a DNaseI control lane were seen.

An indirect approach was therefore taken to detect the protein. Since the sequence of pSCL1 is known (see Figure 1), a restriction enzyme was chosen that cut close to the plasmid termini with very few cuts internally. The purified plasmid was digested with *Asp*718I and the resulting 5' overhangs were filled in with non-radioactive dATP, dGTP, dTTP, and $\alpha[^{32}P]$-dCTP. Half of the pSCL1 *Asp*718I digest was treated with proteinase K. Following this step, the mixtures were heat treated for 5 minutes at 95°C in preparation for SDS-PAGE . The products were then fractionated on a 12% SDS-PAGE (Figure 17). Unincorporated ^{32}P nucleotides ran with the dye front and were

Figure 15: Polyacrylamide gel electrophoresis (12% SDS-PAGE) of *S. clavuligerus* cell lysate extracted with phenol. Samples of the aqueous phases of four successive extractions with phenol (see Figure 5) are shown in lanes 1, 2, 3, and 4. The corresponding phenol phase samples are shown in lanes 6, 7, 8, and 9. The protein content of the interface material is shown in lane 5. Protein size markers, *E. coli* β-galactosiadase (116 kDa), Rabbit muscle phosphorylase (97 kDa), Bovine serum albumin (67 kDa), Ovalbumin (43 kDa), Trypsin Inhibitor (20 kDa), and α-Lactalbumin (14 kDa), are shown in lane 10.

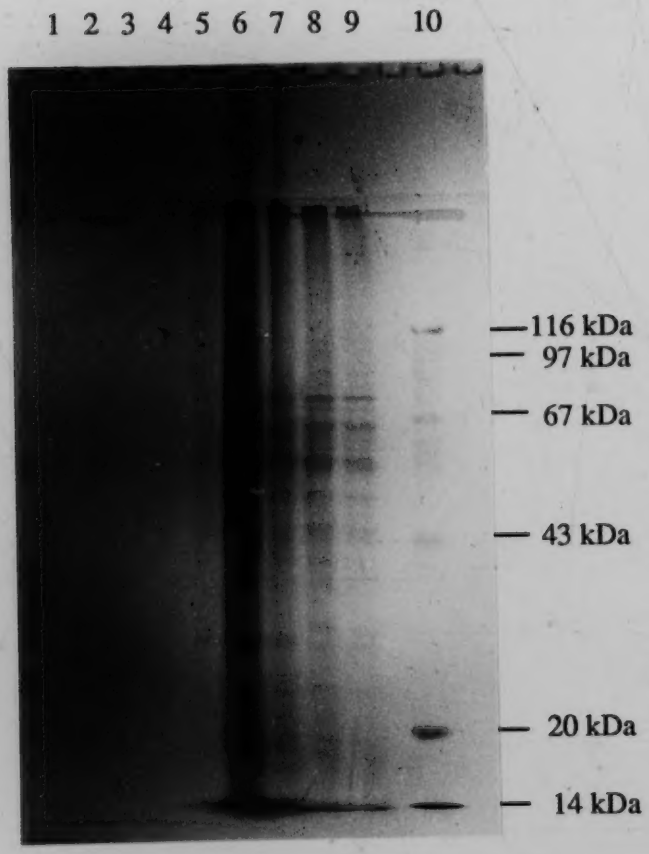

1 2 3 4 5 6 7 8 9 10
116 kDa
97 kDa
67 kDa
43 kDa
20 kDa
14 kDa

Figure 16: Polyacrylamide gel electrophoresis (12% SDS-PAGE) of *S. clavuligerus* cell lysate extracted with phenol:chloroform (1:1, v/v). Samples of the four successive aqueous phases shown in Figure 6 are shown in lanes 1, 2, 3, and 4. The corresponding phenol:chloroform phases are shown in lanes 6, 7, 8, and 9. The proteins remaining in the interface are shown in lane 5. Protein molecular size markers, *E. coli* β-galactosiadase (116 kDa), Rabbit muscle phosphorylase (97 kDa), Bovine serum albumin (67 kDa), Trypsin Inhibitor (20 kDa), and α-Lactalbumin (14 kDa), are shown in lane 10.

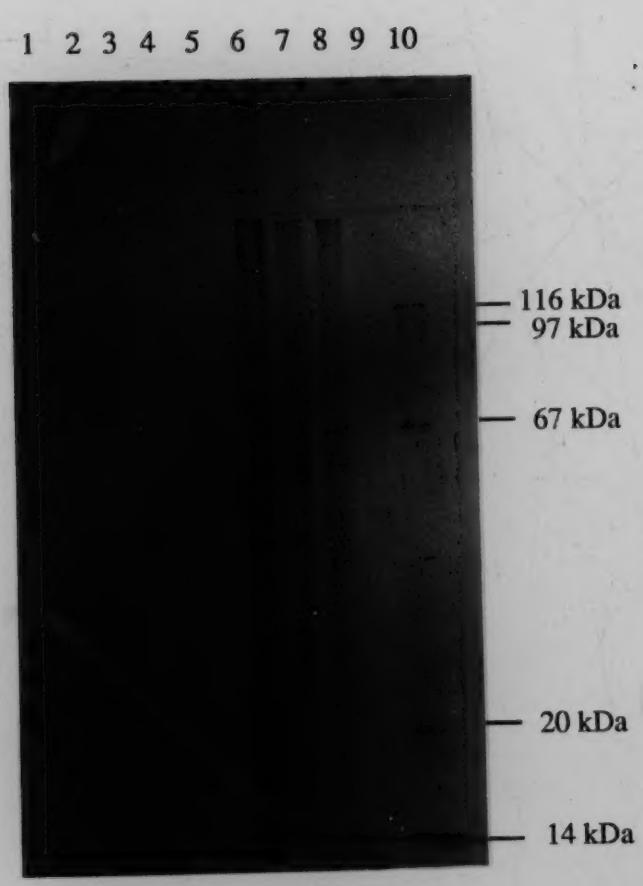
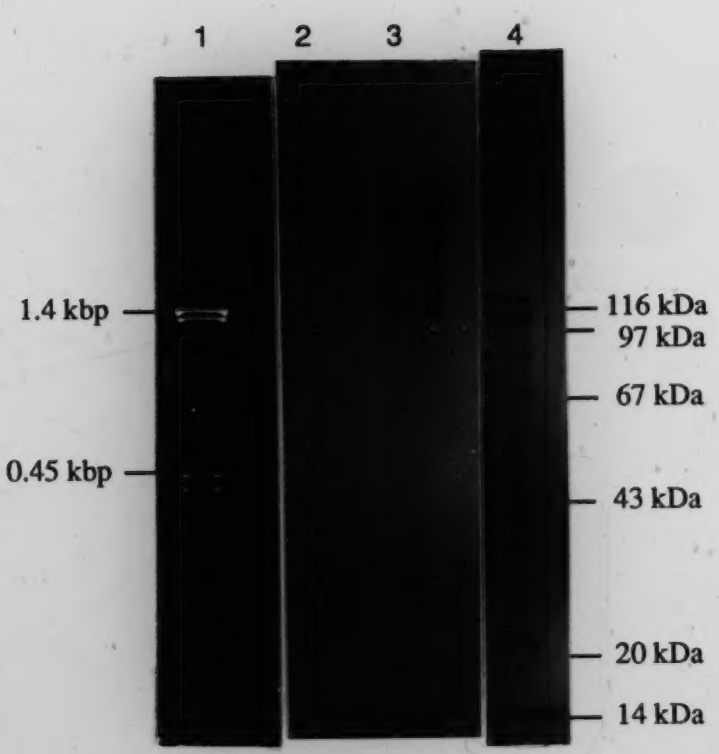

1 2 3 4 5 6 7 8 9 10
116 kDa
97 kDa
67 kDa
20 kDa
14 kDa

Figure 17: Interface isolated pSCL1 digested with *Asp*718I and filled-in with [^{32}P]-dCTP. Lane 2 shows results of proteinase K digestion after fill-in reaction. Lane 3 shows results of the fill-in reaction without proteinase K digestion. pUC118 digested with *Taq*I was run in lane 1 as a molecular size marker. Protein molecular size markers, *E. coli* β-galactosiadase (116 kDa), Rabbit muscle phosphorylase (97 kDa), Bovine serum albumin (67 kDa), Ovalbumin (43 kDa), Trypsin Inhibitor (20 kDa), and α-Lactalbumin (14 kDa), are shown in lane 4. All samples were treated in accordance to techniques for preparing proteins being run on SDS-PAGE.


1
2
3
4
1.4 kbp
0.45 kbp
116 kDa
97 kDa
67 kDa
43 kDa
20 kDa
14 kDa

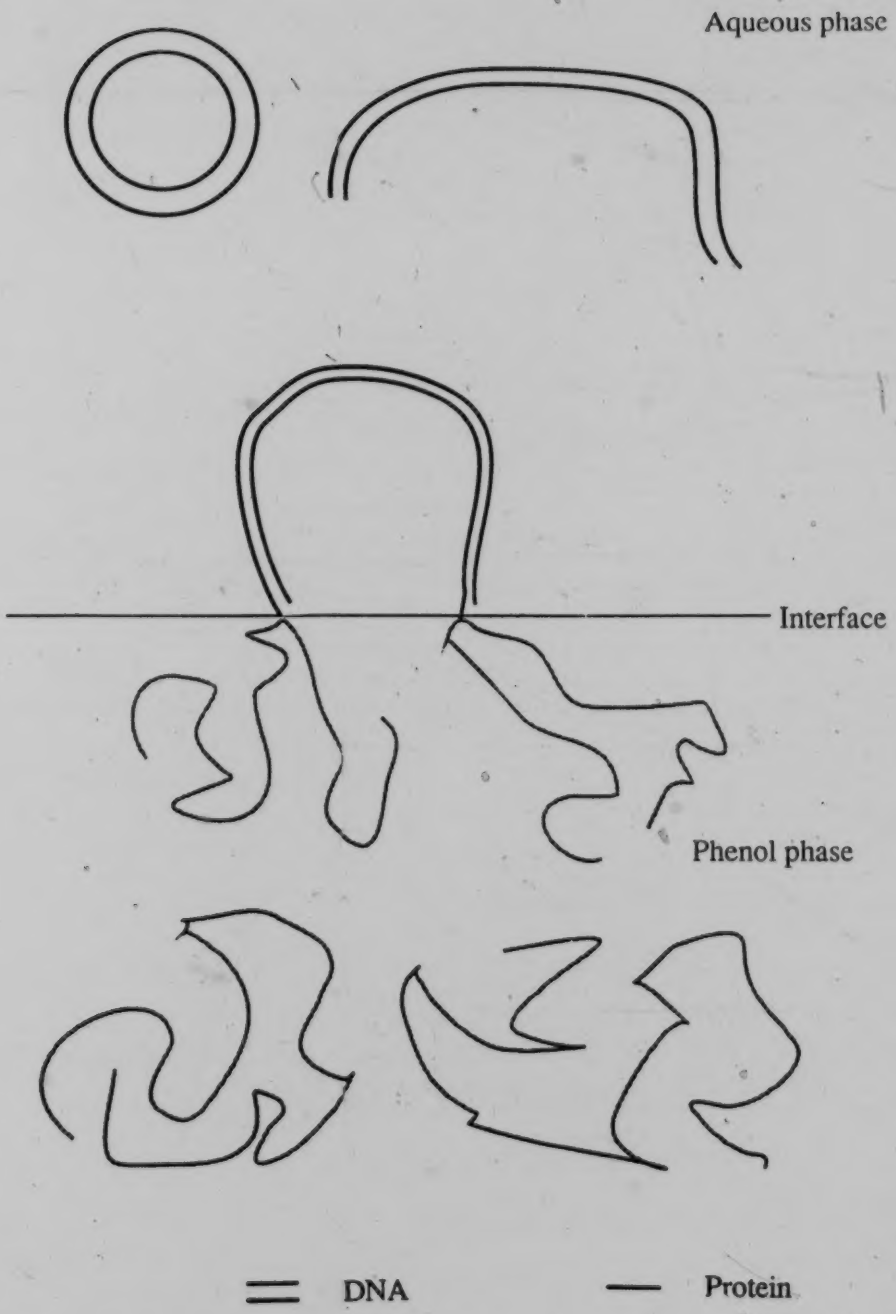
removed from the gel prior to exposure to X-ray film. The autoradiograph obtained clearly showed a number of bands. The band at the top of the gel in each lane corresponds to the sample well. The second band corresponds to the boundary between the stacking and separating gels. A third band visible only in the non-proteinase K treated lane, runs with the mobility of a protein of approximately 97 kDa and a linear DNA fragment of about 1.4 kb. There is also a faint band towards the bottom of the separating gel in each lane. The DNA marker used was pUC118 digested with *Taq* I. It appears that the strong signal given by the third band visible only in the non-proteinase K digested lane corresponds to the 1.4 kb "left" internal fragment of pSCL1. The faint band towards the bottom of the gel most likely corresponds to the terminal 16nt oligonucleotide.

4. DISCUSSION

The objective of this study was to develop a novel method for isolation of linear plasmids of the type found in *Streptomyces*. It is clear that this has been achieved, but further development is still required.

The method relies on the propensity of the covalently bound terminal protein of the linear plasmid to dissolve in phenol during the phenol extraction step. It is not sufficiently widely recognized that most proteins will partition preferentially into the organic phase during phenol extraction. Nucleic acids, being polyanions, remain in the aqueous phase. As discussed earlier, protein tertiary structure is disrupted by phenol when phenol molecules interact with both the hydrophobic and hydrophilic regions. Phenol disrupts the protein to such an extent that it can interact with the hydrophobic core of the protein. This disruption causes proteins to unfold. Unfolding of proteins changes their conformation and causes them to become less soluble in water. Proteins are, however, highly soluble in phenol. The vigorous mixing during the extraction step allows the phenol to interact with most of the proteins in the aqueous phase, dissolving almost all proteins. The DNA, on the other hand, remains dissolved in the aqueous phase. The mixing forms an emulsion of buffer/cell lysate and phenol which, when centrifuged, separates into the two phases. Linear plasmids can be found at the boundary between the aqueous and the phenol phases in the insoluble material that collects there, with most of the protein in the phenol phase and most of the DNA in the aqueous phase (Figure 17). In other DNA and RNA isolation methods the aqueous phase is physically removed to be processed further. The interface and the phenol phase are discarded. Some small amount of the aqueous phase remains with the

Figure 18: Diagrammatic representation of possible molecular content of aqueous and phenol phases after phenol extraction of cell lysate followed by centrifugation to separate the two layers. Both chromosomal DNA and cccDNA plasmids are found solely in the aqueous phase, whereas proteins are found in the phenol phase. Linear plasmids, such as pSCL1, are found at the interface, with the DNA portion in the aqueous phase and the protein portion of the holoplasmid in the phenol phase.


Aqueous phase
Interface
Phenol phase
DNA
Protein

discarded material because it is physically impossible to remove the complete aqueous phase. For this reason, the linear plasmid becomes part of the material normally discarded and is therefore not detected.

In this new method the two phases are physically removed, separately, from the interface. Macromolecules in the normally discarded interface material are precipitated with ethanol, thus removing residual phenol. Intact linear plasmids, perhaps with other unidentified macromolecules, are left in the precipitated material. Linear plasmids can then be redissolved, to some extend, using buffer and a detergent such as SDS. The detergent allows the protein moieties to become soluble in buffer again. This is exactly what has been demonstrated in the first part of the Results (Figure 5). It is seen that the aqueous phase has been cleared of the linear plasmid, presumably because of the interaction between the proteins and phenol. The aqueous phase contains no detectable protein (Figure 15). The phenol phases, as expected, contain no DNA. The linear plasmid is clearly detectable at the interface.

The results obtained for phenol/chloroform extraction, a variation of the phenol extraction method, are similar to those observed with phenol. Chloroform is an organic solvent insoluble in water but miscible with phenol. It is used mainly to increase the density of the organic phase, promoting easier phase separation, and also to remove residual phenol from the aqueous phase, as that might interfere with later DNA manipulations. Proteins are not as readily soluble in chloroform, so the mixture of phenol:chloroform (1:1, v/v) is initially used to denature proteins and to remove them from the aqueous phase. The chloroform also reduces the amount of phenol in the aqueous phase.

In an effort to identify in which phase the proteins are located after phenol or phenol:chloroform extractions, samples from each of the aqueous

phases and organic phases, in addition to the interface, were electrophoresed on 12% SDS-PAGe. This was done for both the pure liquefied phenol and also the phenol:chloroform treatments. In either case, the results show that the vast majority of proteins are dissolved in the organic phase. There are some proteins present in the interface, however, this might be explained by the fact that there was likely some residual organic phase present when the interface material was precipitated with ethanol. TPs are present as well, however, they are still covalently bound to the plasmid which cannot enter the separating gel. In addition to this, they are present in very low amounts and therefore probably not detectable using Coomassie Blue stain. The interface prepared with phenol:chloroform also showed some proteins present, but the difference between the two preparations is minimal. It is thus clear that phenol, in the absence or presence of chloroform, dissolves proteins and removes them from the aqueous phase.

Equilibration of phenol with buffers has been widely used, putatively to prevent a major loss of DNA from the aqueous phase and into the phenol phase (Sambrook *et al.*, 1989). These results demonstrate that this does not happen. When using non-equilibrated phenol, no DNA is found in the organic phase. Some DNA is in the interface, but this is likely due to incomplete removal of the aqueous phase from the interface and phenol phase as well as trapping of protein-linked DNA at the interface. Because *Streptomyces* have linear plasmids and also a linear chromosome, the end fragments of these genetic elements will likely be at the interface explaining the presence of some or all of the DNA at the interface. The amount of the interface material appeared diminished with an increase of pH of the equilibrated phenol. This may be due to more efficient partitioning of the

protein into the phenol phase at higher pH. Equilibration seems to affect the volume of the aqueous phase more than the amount of DNA "moving out" of the aqueous phase. Equilibrated phenol is already water saturated and will not be able to take up more water from the aqueous phase. Non-equilibrated phenol, when mixed with a cell lysate, will take up water from the aqueous phase, reducing the volume of the aqueous phase. A conclusion that can be drawn from this is that when working with small volumes the use of equilibrated phenol is highly desirable so as not to reduce the volume any further and thereby lose too much of the DNA. The loss of DNA is mainly due to the relative percentage of the aqueous phase being left behind as a result of attempts to prevent the DNA fraction from being contaminated with the interface material and phenol. The actual loss does not appear to be due to the DNA moving out of the aqueous phase, but to reduced volume, making a more complete recovery of DNA more difficult.

The linear plasmid concentrated from the interface material is not readily soluble in buffer. The protein appears to form complexes (possibly hydrophobic) with other molecules in the interface that prevent the plasmid from moving from the precipitated interface material into the redissolving buffer. Even though DNA makes up most of the plasmid (probably >99%), it is not sufficient to solubilize the plasmid. It has to be noted that at this stage most or all of the phenol has been removed from the interface material by ethanol precipitation. Therefore, there is no phenol present which might force the plasmid to remain with the precipitated interface material.

To obtain good recovery of pSCL1 from the interface material, these interactions needed to be disrupted. For this purpose, several redissolving agents were tested. SDS, a strong anionic detergent, works moderately well in

disrupting these interactions to allow the plasmid to be released. It binds to the protein, thereby possibly disrupting the hydrophobic interactions. This allows the protein to become dissolved in the buffer.

Proteinase K, an enzyme that works well at high temperatures (55°C) in the presence of SDS, gives good recovery of the linear plasmid. This enzyme is a non-specific protease that can digest most proteins almost completely. Because it digests proteins extensively the majority of the plasmid molecules are released in a protein-free form into the buffer. All the hydrophobic interactions due to the protein are now destroyed. However, this can create a problem. If only the DNA component of the plasmid is required, proteinase K can be used to give maximum recovery. On the other hand, if the target is the terminal protein or the holoplasmid (complete linear plasmid) it is completely lost and no further studies can be done. The SDS-proteinase K treatment works well, however, as a control to show that the linear plasmid is in the interface material.

Other chaotropic agents were tested as well. Urea, for example, is known to be a good denaturing agent of proteins. It also has been used for many years to reduce or eliminate secondary structure in nucleic acids (Tomlinsen and Tener, 1962). It is a mild agent that disrupts hydrogen bonds and hydrophobic interactions. It is very successful in disrupting the bonds formed in the interface material, leading to release the linear plasmid. In fact, it gave as good a recovery as using proteinase K. The physical appearance of the interface material was also changed dramatically by urea, indicating its effectiveness. The success of this agent was not immediately recognized and therefore it was not used to its fullest extent. This occurred because its efficient solubilization led to overloading of agarose gels when samples

redissolved with urea were electrophoresed and no distinct pSCL1 band was observed.

Two other reagents, guanidinium hydrochloride and guanidinium thiocyanate, were also tested. They were also effective in changing the appearance of the white interface precipitate to a more clear state. Both guanidinium salts are powerful chaotropic agents and act by disrupting ionic and hydrogen bonds, as well as hydrophobic interactions. In fact, they are much stronger agents than urea, but they don't appear to be as useful since recovery of the linear plasmid was much lower. As Figure 8 has shown, the linear plasmid could not be detected at the same level as when urea or SDS:proteinase K was used. Samples treated with either guanidinium salt had to be precipitated with ethanol and redissolved in TE buffer prior to electrophoresis. This is an unavoidable consequence of the very high ionic strength of guanidinium salt solutions, which prevent electrophoretic separations. This extra step is very likely responsible for the greatly reduced yield of pSCL1 from the interface using these agents. With the removal of the guanidinium salts by ethanol precipitation, the TPs are able to interact with other redissolved compound from the interface. These interactions can likely cause the reduced recovery of the holoplasmids.

Even though this precipitation step was also used when urea was used as a redissolving agent it did not appear to hinder pSCL1 recovery. Because urea is much milder treatment, it might not have had the same affect on the protein and other compounds as the guanidinium salts. It is possible that the guanidinium salts are so good at disrupting the bonds that they completely unfold the protein to such an extent that dissolution in TE buffer after precipitation with ethanol is not possible under the conditions used. The most

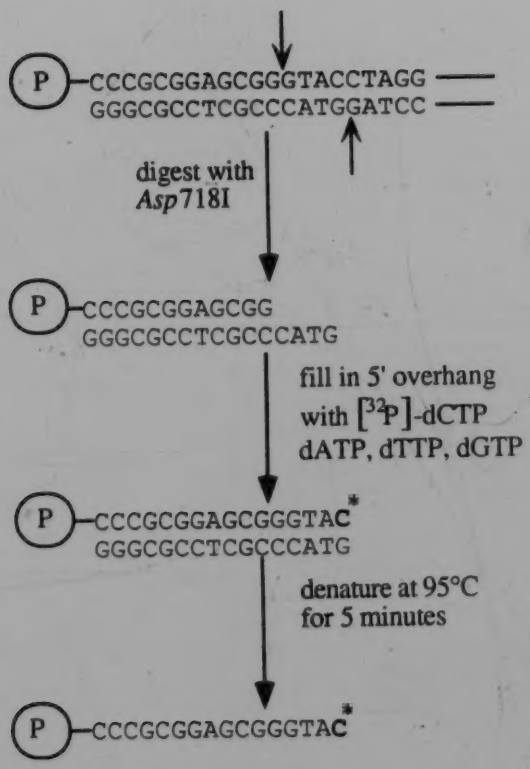
effective treatment for recovery of holoplasmid and other protein-linked DNA (and probably RNA) molecules from phenol-aqueous interfaces would likely be dissolution in 7 to 8 M urea.

One of the objectives in studying the protein-linked holoplasmid was to identify and possibly isolate this protein. Direct detection using Coomassie Blue to stain proteins in the polyacrylamide gels was attempted but proved to be unsuccessful. Calculation of the amount of DNA and protein present, however, clearly explains that failure. pSCL1 is a 11.7 kbp linear plasmid. This is equivalent to 7.6×10^{-6} Da of DNA. Since 1 dalton is the weight of a hydrogen atom (1.66×10^{-24} g) 1 pSCL1 molecule is the equivalent of 12.6×10^{-12} µg. Also, 1 pmol of 1000 bp DNA is equal to 0.66 µg. Therefore, 1 pmol of pSCL1 is equal to approximately 7.7 µg. The amount of DNA present in a strong ethidium bromide stained band shown in some of the figures in the Results might be about 50 ng or about 6.5 fmol. For each plasmid, there are two proteins, so there are 13 fmol of protein per 6.5 fmol of plasmid. For purification purposes, approximately 200 µL were loaded on a sucrose gradient. After fractionation of the gradient each precipitated fraction was redissolved in 20 µL of TE Buffer. Two µL were electrophoresed on a gel, showing at most 50 ng of the plasmid in the appropriated fraction. In the remaining fraction, there would be 450 ng of DNA, or 0.06 pmol. This is equivalent to 0.12 pmol of protein. Assuming a 20,000 Da protein, the amount of protein would be about 2.4 ng, well below the level of detectability for Coomasie Blue. In retrospect, some of the other staining procedures available should have been applied. Silver stain might have detected the terminal protein band, but because of the staining process, it would have been very difficult to use this for any further manipulation. A copper stain or fluorescent

dye, now available, might also have detected the protein. Either one of these stains is reversible, thereby allowing further manipulation after the stained band is cut out and the protein eluted.

A different approach to identifying the protein was taken, though. Since small volumes and amounts were usually dealt with, the best approach seemed to be a radioactive labeling experiment. Since the sequence of pSCL1 is known (Figure 1) the best way to identify the protein seemed to be an indirect labeling method. The restriction endonuclease *Kpn*I is known to have three cleavage sites on pSCL1. Of these, two are very close to, and equidistant from, the termini. The third site is located at about 1.4 kbp from the left hand side (Figure 1). These three sites produce four fragments of known sizes. *Asp*718I, an isoschizomer of *Kpn*I, produces a 5' overhang which can be filled in with $\alpha[^{32}P]$-dCTP, thereby labeling the end. A diagrammatic representation of this is given in Figure 19. After filling in the overhangs, the reaction mixture was treated in the same manner as proteins are treated prior to electrophoresis on a polyacrylamide gel. This involves addition of β-mercaptoethanol in the sample buffer and heating at 95°C for 5-10 minutes. If this is then run on a 12% SDS-PAG, three distinct bands should be observed on the autoradiograph. The two large fragments, 1.4 kbp and 10.3 kbp, should have molecular weights of 473.2 kDa and 3.3 MDa respectively. The other two fragments of 16 bases plus the protein should have identical sizes of 5.2 kDa plus the size of the protein. It has to be considered here that the 16 bp fragment created during the digestion and fill in step would most likely have been denatured during the heating step leaving only a 16 base labeled fragment covalently attached to the protein. The complementary 16 base oligonucleotide is not labeled and therefore would not be detected. It was

Figure 19: Diagrammatic representation of the fill-in experiment performed. One terminal fragment of pSCL1 is shown. The cut site of *Asp*718I on pSCL1 is indicated with the arrow and the radioactive nucleotide is shown by the bold letter with the star above it.


P
CCCGCGGAGCGGGTACCTAGG
GGGCGCCTCGCCCATGGATCC
digest with
Asp718I
P
CCCGCGGAGCGG
GGGCGCCTCGCCCATG
fill in 5' overhang
with [32P]-dCTP
dATP, dTTP, dGTP
P
CCCGCGGAGCGGGTAC*
GGGCGCCTCGCCCATG
denature at 95°C
for 5 minutes
P
CCCGCGGAGCGGGTAC*

observed that there were three distinct bands of radioactivity, one at the border between the stacking and resolving gel and two in the resolving gel. A very faint band was observed at the top of the well, representing material that did not move into the gel. The band at the border between the two types of gels is likely the 10.3 kbp large DNA fragment that could not move beyond this point since it was too large to enter the 12% gel. The band running at the mobility of a protein of approximately 97 kDa is the 1.4 kbp "left hand end" fragment of the plasmid. At first it was thought that this large fragment would not be able to move into the 12% separating gel, however, the control lane shows that this band is indeed the 1.4 kbp pSCL1 fragment. pUC118 was digested with *Taq*I and run on a 12% SDS-PAG under the conditions used for protein separations. A 1.4 kbp DNA fragment ran at the same rate as the radiolabelled band of the autoradiograph. Also, the radiolabelled pSCL1 lane was stained with ethidium bromide and the band in the separating gel appears to correspond to the 1.4 kbp fragment (data not shown). A linear 1.4 kbp DNA fragment (or 470 kDa), therefore, moves at the same rate as a protein of approximately 97 kDa. DNA mobility on a 12% SDS-PAG is clearly very different from that of proteins on the same gel.

A faint band near the bottom of the gel is likely to be the small 16 base DNA fragment that was covalently attached to the terminal protein. This band is not a result of unincorporated $\alpha[^{32}P]$-dCTP, as this nucleoside triphosphate ran much faster and was excised prior to exposure to X-ray film. As a control, the *Asp*718I digested pSCL1 was treated with proteinase K after ^{32}P fill-in reaction. It was hoped that this would allow an estimation on the size of the protein, as there should be migrational differences between the end fragments with or without a TP. This portion of the experiment proved unsuccessful, as

the band corresponding to the 1.4 kbp fragment disappeared completely, without any new bands appearing.

Of the possible explanations none appear to correspond with the obtained data. First, it was hypothesized that there was incomplete digestion of the linear plasmid by the restriction enzyme. If this is the case, it would most likely be that the restriction sites at the termini would be most affected as the presence of the TP could possibly interfere sterically with the restriction enzyme. Once the restriction fragments are labeled and then digested with proteinase K, the 1.4 kbp linear fragment would still have to be present. Also, if the terminal *Asp*718I sites were blocked the small, 16 base fragment should have a much lower intensity, if not be completely absent, since it would have not been labeled.

Secondly, the 1.4 kbp fragment was properly identified. In this experiment pUC118 was digested with *Taq*I and used as a marker. The fact that a 1.4 kbp dsDNA fragment would migrate as fast as it did in a 12% SDS-PAG came as a complete surprise. Since a similar lane with a pSCL1 *Asp*718I digest was also ethidium bromide stained and the 1.4 kbp fragment was obvious and had a similar mobility, there can be no question about the identity of this band.

I considered the possibility that the TP might be so small that it could not be detected using this particular gel system. This cannot explain the disappearance of the 1.4 kbp fragment. The possibility of a very small protein cannot be rejected yet, however, this would not explain the identical migration rates of the 16 base fragment.

Finally, the most likely scenario at present is the possibility that the phosphodiester bond between the β-hydroxyl of the amino acid and the first

nucleotide is broken during the processing of the DNA:protein conjugate in preparation for running on an SDS-PAG. The heating step may break this bond. This would certainly prevent the detection of the TP. However, this also does not explain the disappearance of the 1.4 kbp fragment in the presence of proteinase K. The latter proposal could explain the presence of the small labeled fragment close to the dye front in both lanes. This band is present in both treatments and can only be ascribed to a small DNA fragment, likely the 16 base fragment created during the digestion of pSCL1.

Direct isolation with stains does not seem feasible, as commercial enzymes, such as DNaseI, are surprisingly impure and their staining profile would likely obscure a band due to the TP. Ultrapure DNaseI is available, but was not considered at this stage. Also, two dimensional polyacrylamide electrophoresis could have been performed, especially with the lower grade DNaseI. This procedure is very effective in separating large number of different proteins. This particular procedure was unfortunately also not considered. Biotinylation is another good approach that could be taken in identifying linear plasmid TPs. Other approaches that could have been taken would have been labeling of the protein either by growth medium containing amino acids with ^{35}S, or using a direct protein iodination labeling with ^{125}I. One concern with the use of ^{35}S is the large amounts of radioactive waste created this way. ^{125}I on the other hand presents a much greater potential health hazard.

Once it was shown that this new method worked well for pSCL1 it was of interest to determine whether it could be applied to linear plasmids in general. For this, other *Streptomyces* species were studied. *S. jumonjinensis*, for example, is known to have two small linear plasmids, pSJL1 and pSJL2, 11.7

and 17.5 kbp in size (Netolitzky *et al.*, 1997). Cultures of *S. jumonjinensis* obtained from two different sources, from the same original isolate, demonstrated different plasmid profiles (Netolitzky *et al.*, 1997). Both plasmids were recovered using this method. Even though the bands are faint the method has proven to be successful with this *Streptomyces* species. Since this study was completed prior to the demonstration of the efficiency of urea as a solubilizing reagent it may be even more successful with 7 M urea solubilization.

S. lividans is one of the best studied and characterized *Streptomyces* species. Several strains of *S. lividans* exist which have been created by Kieser and Hopwood (Hopwood *et al.*, 1983). A few of these *S. lividans* strains were tested to see if the extraction method could be applied to the genus *Streptomyces* in general. The wild type strain of this species, 1326, has two plasmids, SLP2 and SLP3, of which only one, SLP2, has been isolated and characterized (Chen *et al.*, 1993). SLP2 is a 50 kbp linear plasmid which has some similarities to the linear chromosome. A transposon is located on it which is also found on the linear chromosome. Figure 12 shows a typical result obtained using this new method of isolation. SLP2 can be readily identified in total DNA preparations run on conventional 0.4% agarose gels, but it is not evident in interface preparations. Because the interface has been treated with SDS, a band should have been visible just below the chromosomal DNA. Since this is not the case, something else has to be the cause for the absence of the linear plasmid.

When it became apparent in the course of this study that urea is also a useful redissolving agent, the *S. lividans* 1326 interface material was subjected to a variety of treatments. Only in the presence of SDS and proteinase K did a

band, similar in size to the linear plasmid SLP2, become apparent. However, even this particular band cannot clearly be identified as SLP2. It is not defined enough on the gel to be certain about its identity. Urea, which proved to be such a good redissolving agent for pSCL1 was not sufficient in this case to allow complete dissolution. This is unfortunate, as the phenol-extraction method can apparently not be used to isolate SLP2 so that the terminal protein can be studied. It might, however, be possible to adjust certain conditions, such as incubation time of the interface with urea, to study the effect on proper dissolution. If that should be the case, it would certainly show the versatility of this newly developed method for isolating linear plasmids.

Certain strains of *S. lividans* have been reported to harbour another plasmid, SLP3 (Hopwood *et al.*, 1983). This plasmid has only been identified by genetic means, pock formation in strains lacking this plasmid when crossed with strains containing the plasmid, but never physically detected or isolated. With this in mind, it was thought that perhaps this new isolation method could help in physical detection of the plasmid. *S. lividans* TK19, a strain reportedly lacking SLP2 but not SLP3 was studied. The results obtained were shown in Figure 14. The aqueous phases show the presence of two plasmid bands, both most probably circular. The interface and the total DNA preparation show a third and fourth band, migrating somewhat faster than the ones in the aqueous phases. There also appears to be a band running slightly faster than the band where sheared chromosomal DNA runs in the total DNA preparation. This band appears to be about 50 kbp in size. This might indicate that this strain contains SLP2. TK19, however, was reported to have been cured of SLP2. This immediately suggests that the strain obtained might have been contaminated by an SLP2 containing strain. To be certain that this is SLP2,

hybridization experiments should be performed using authentic SLP2 from strain 1326 as a probe. The question remains, where has the contamination occurred, as conjugation experiments were never performed in this laboratory and the strain obtained was always kept separate from either *S. lividans* 1326 or TK20.

The other four plasmids that seem to be present in this strain also have to be studied further. The fact that two of these bands are only detected when proteinase K is used prior to extraction with phenol, or they are detected using the phenol-interface extraction method indicates they have a protein covalently attached or extremely firmly bound, and therefore could possibly be linear. However, the two plasmids were examined by electron microscopy (Netolitzky, 1995) and shown to be circular. This demonstrated that the previously hypothesized linear topology was incorrect. Further investigations into the identity of these plasmids have to be done. This could include hybridization using known *Streptomyces* vectors such as pIJ702 or pIJ941. Closer examination of the two lower bands in the total DNA preparation lane shows that these two bands are actually doublets, thereby further complicating the study of these plasmids.

In conclusion, the newly developed method has proved to be very effective in concentrating certain linear plasmids. The methodology relies on the differential response of the macromolecules to the phenol. After cells are lysed, the cell lysate is exposed to liquefied, unequilibrated phenol. This is usually done by vigorous mixing which allows most or all of the proteins to interact with phenol. The proteins at this point become denatured and dissolve in the phenol. This step is highly desirable, as it eliminates most or all of the enzymes responsible for the degradation of the DNA or RNA.

Separation of phenol from the aqueous phase occurs through centrifugation. This resolves the emulsion into two phases separated by the interface. Both phases are removed separately and several more extractions are performed on the interface, to remove as much phenol-soluble and water-soluble material as possible. Once both phases, aqueous and phenol, have been removed as completely as possible, macromolecules remaining at the interface are precipitated using ethanol. The precipitated interface material is redissolved in buffer using solubilizing agents such as SDS or urea. These reagents disrupt interactions between different macromolecules in the interface and allow at least some of the holoplasmid to dissolve in the buffer. At this stage the plasmid can then be further manipulated, depending on what needs to be investigated further.

The development of this method was done using large quantities of mycelium (usually several grams wet weight), and therefore large volumes were manipulated. To allow for further use of this method, it would need to be scaled back so that it could be applied without the use of large cell masses. This refinement of the method could make it much more useful for investigations of linear plasmids, especially with respect to screening.

Another refinement of this method could be made so that it could be done at the same time as conventional DNA isolation methods. The aqueous phase of a phenol extraction can be used for common DNA investigations, while the interface could be investigated for unusual DNA molecules having forms similar to pSCL1.

New research using this method could be initiated to determine the most successful solubilizing agents for the linear plasmids. It has been shown here that SDS and urea are successful in disrupting bonds within the interface to

allow the plasmid, and possibly other macromolecules, to dissolve in buffer. Other agents, not used in this study, should be tested to assess their effectiveness in plasmid recovery. Also, investigations into reasons for the failure of the guanidinium salts with respect to plasmid recovery should be initiated. Since both salts changed the interface appearance dramatically they are probably both very effective in disrupting bonds formed in the interface precipitate, but later steps prevent efficient recovery. If these steps can be circumvented, it could increase plasmid recovery.

One study that should be initiated is a more extensive examination of the components of the interface. The holoplasmid is located at the interface, as are some proteins, but the question arises whether other molecules are located here and what their identity might be. Also, are these other components the reason why a solubilizing agent has to be included to enhance plasmid recovery?

Certainly the terminal protein has to be investigated further to understand replication in *Streptomyces* more completely. The size of the terminal protein is not yet known. If other staining systems are used, the position of the protein on SDS-PAGE might possibly be detected. In addition, enough of the protein might be obtained for N-terminal sequencing so that investigations into the terminal protein gene could be initiated. Once the gene has been identified, various aspects of expression of the protein could be studied. Also, the location of the gene could then be determined to study the replication process, not only in *S. clavuligerus*, but probably in the whole genus.

Since this method is mainly selective for DNA:protein conjugates, the application of this method to the termini of the linear chromosome is feasible.

The *S. lividans* linear chromosome has the same structure as SLP2, a linear plasmid. So the termini of the chromosome are likely to be present in the interface and should be easily recovered for further study. This could be applied to other *Streptomyces* species as well, however, this might be complicated by the presence of GLPs such as the ones found in *S. clavuligerus* and *S. jumonjinensis*.

Another question raised by the investigation concerning TK19 is the presence of a 50 kbp DNA band in the total DNA preparation. This immediately suggests contamination of the strain obtained by a SLP2 (50 kbp in size) containing strain. However, this is not necessarily a certainty. To be sure, this will have to be investigated using probes specific for SLP2. This might be complicated because SLP2 contains Tn4811, a transposon also present on the *S. lividans* chromosome. Is it possible that this is actually SLP3, and that this is the reason why it hasn't been physically identified? If SLP3 had a size very similar to SLP2 it might provide a reason why it hasn't yet been identified. *B. burgdorferi* strain B31 has just recently been shown to have four linear plasmids of similar size. They could only be distinguished when the sequence of each was obtained (Fraser *et al.*, 1997). This certainly raises the possibility that this phenomenon might also occur in *Streptomyces*.

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